

A Novel Class of Antitumor Prodrug, 1-(2'-Oxopropyl)-5-fluorouracil (OFU001), That Releases 5-Fluorouracil upon Hypoxic Irradiation

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We have been developing prodrugs of anticancer agents such as 5-fluorouracil (5-FU) that are activated by irradiation under hypoxic conditions via one-electron reduction. Among them, OFU001 [1-(2'-oxopropyl)-5-fluorouracil] is a prototype radiation-activated prodrug. In this study, we investigated the radiation chemical reactivity and the biological effects of OFU001. This prodrug is presumed to release 5-FU through incorporation of hydrated electrons into the antibonding σ^* orbital of the C(1')-N(1) bond. Hydrated electrons are active species derived from radiolysis of water, but are readily deactivated by O₂ into superoxide anion radicals (O₂⁻) under conditions of aerobic irradiation. Therefore, 5-FU release occurs highly specifically upon irradiation under hypoxic conditions. OFU001 dissolved in phosphate buffer released 5-FU with a *G*-value (mol number of molecules that are decomposed or produced by 1 J of absorbed radiation energy) of 1.9×10^{-7} mol/J following hypoxic irradiation, while the *G*-value for 5-FU release was 1.0×10^{-8} mol/J following aerobic irradiation. However, the *G*-values for decomposition of OFU001 were almost the same, i.e., 3.4×10^{-7} mol/J following hypoxic irradiation and 2.5×10^{-7} mol/J following aerobic irradiation. When hypoxically irradiated (7.5–30 Gy) OFU001 was added to murine SCCVII cells for 1–24 h, a significant cell-killing effect was observed. The degree of this cytotoxicity was consistent with that of authentic 5-FU at the corresponding concentrations. On the other hand, cytotoxicity was minimal when the cells were treated with aerobically irradiated or unirradiated OFU001. This compound had no radiosensitizing effect against SCCVII cells under either aerobic or hypoxic conditions when the drug was removed immediately after irradiation. Since hypoxia is generally most marked in tumors and irradiation is applied at the tumor site, this concept of prodrug design appears to be potentially useful for selective tumor treatment with minimal adverse effects of anticancer agents.

Key words: Prodrug — Radiation-activated — 5-Fluorouracil — Radiation — Hypoxia

Anticancer agents are used to control local tumors and/or to prevent systemic metastasis. For the former purpose, an ideal method is to administer the drugs to tumors topically. Currently, most anticancer agents are given systemically, and their adverse effects are unavoidable. Regional infusion is a method of locally applying anticancer agents, but even with this method, systemic effects are not negligible. Another method of treating tumors efficiently by decreasing damage to normal tissues is the use of prodrugs. Most of the previously developed prodrugs are activated *in vivo* through enzymatic processes.^{1–4} If the appropriate enzymes are more abundant in tumor tissue or can be delivered to a tumor using monoclonal antibodies, selective treatment of tumors becomes possible. Recently, a new method of activating prodrugs, i.e., enzyme prodrug gene therapy, is attracting more attention.^{5–7}

Another novel class of tumor-specific prodrugs which we describe here is activated by hypoxic irradiation. In

their previous study, Nishimoto *et al.*⁸ found that an N(1)-C(5)-linked dimer of 5-fluorouracil (5-FU) was converted to 5-FU following irradiation. This conversion occurred more efficiently when the dimer was irradiated under hypoxic conditions than when it was irradiated under aerobic conditions. Radiation is intended to be applied to tumor sites only, and in addition, hypoxic cells are thought to be present only in tumors. Therefore, this type of prodrug appeared promising in terms of selective tumor treatment. The conversion of the 5-FU dimer into 5-FU was not so efficient and not so specific to hypoxic conditions,⁸ so no further investigation of this compound was performed. Instead, we searched for various other compounds that release 5-FU in response to hypoxic irradiation through a similar mechanism.

Recently, we have found that a series of compounds with 2-oxoalkyl side chains at the N(1)-position of 5-FU are converted efficiently to 5-FU following hypoxic irradiation.⁹ Among approximately 30 compounds synthesized and tested, 1-(2'-oxopropyl)-5-fluorouracil (OFU001) is a prototype compound in terms of the simplest side-chain

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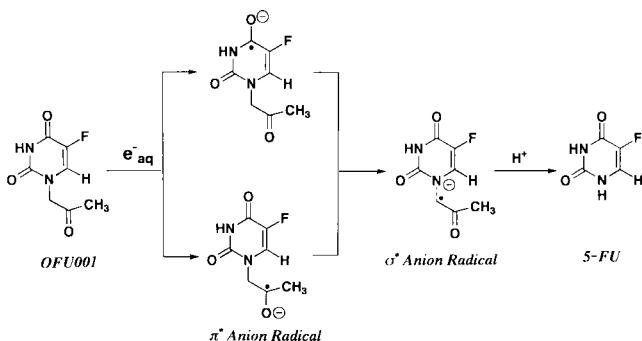


Fig. 1. Proposed mechanism for conversion of OFU001 into 5-FU. e^-_{aq} , hydrated electron. See text for detailed explanation.

structure. The proposed mechanism of 5-FU release by irradiation is shown in Fig. 1. Hydrated electrons (e^-_{aq}) are thought to be incorporated into the compound to form the corresponding π^* anion radical, which is thermally activated to the σ^* anion radical with a weakened C(1')-N(1) bond. Subsequently, hydrolytic dissociation of the C(1')-N(1) bond occurs, releasing 5-FU. In this study, we investigated the radiation chemical reactivity and the *in vitro* antitumor effect of OFU001.

MATERIALS AND METHODS

Compound This compound has been reported by a Korean group¹⁰⁾ as one of a series of acyclic 5-substituted pyrimidine nucleoside analogues. For this study, we synthesized OFU001 by reaction of 2,4-bis(trimethylsilyloxy)-5-fluoropyrimidine (1.21 g) and bromoacetone (5.0 ml) in acetonitrile under an N_2 atmosphere. After 1 h of refluxing, 1.0 ml of methanol was added to the reaction mixture, and the mixture was stirred for 30 min at room temperature. The product was separated and purified by silica gel column chromatography (yield 53%) and identified by nuclear magnetic resonance and high-resolution mass spectroscopy. OFU001 has a molecular weight of 186. The partition coefficient in octanol/water measured by a published method¹¹⁾ was 0.067. As a reference compound, 5-FU obtained from Tokyo Kasei Organic Chemicals (Tokyo) was also used.

Radiation chemistry To measure radiolytic decomposition of OFU001 and release of 5-FU, OFU001 dissolved in phosphate buffer (pH 7.0) was irradiated with an X-ray apparatus (250 kVp, 15 mA, 1.0 mm Al filter) at a dose rate of 5 Gy/min under aerobic or hypoxic conditions at room temperature. To establish hypoxia, Ar gas was bubbled for 15 min according to a published method¹²⁾ and the test tubes were then sealed. Immediately thereafter, the solution was subjected to high-performance liquid chroma-

tography (HPLC) analysis. HPLC was performed using a NANOSPACE SI-1 (Shiseido, Tokyo) equipped with a UG120 C18 semimicro-column (1.5 mm ϕ ×250 mm), and the elution peaks were detected using a UV-VIS detector 2002 at 264 nm wavelength. Phosphate buffer containing 1% methanol (pH 3) was used as an eluent.

***In vitro* effects** Exponentially growing murine SCCVII cells with a doubling time of 14 h were used. The cells were cultured in Eagle's minimum essential medium (MEM) containing 12.5% fetal bovine serum. In all experiments, colonies containing 50 or more cells were counted after fixation on day 7 following drug treatment. The control plating efficiency of this cell line was 63 ± 7 (SD) % during the experiments. To investigate the cytotoxicity of irradiated and unirradiated OFU001, 250 SCCVII cells were plated onto each culture dish 7.5 h before treatment with the drugs. MEM containing 1 mM OFU001 was irradiated with 7.5–30 Gy under aerobic or hypoxic conditions. To make the drug-containing medium hypoxic, it was bubbled with 95% N_2 /5% CO_2 gas for 15 min and then the flask was sealed. After irradiation with 4 MV X rays at a dose rate of 5 Gy/min, the drug-containing MEM was supplemented with fetal bovine serum at 10%, and then the medium in the dishes containing SCCVII cells was replaced with drug-containing medium. After 1, 3, 6, 12, or 24 h, the drug-containing medium was discarded, and the cultures were washed once with phosphate-buffered saline, and supplemented with fresh medium. For controls, MEM containing no drug was also bubbled with the gas, irradiated, and added to control cell cultures; bubbling and irradiation of MEM did not change the plating efficiency of SCCVII cells. Also, one group of cell cultures was treated with unirradiated OFU001 after bubbling with the gas.

The cytotoxicity of 5-FU was also investigated using SCCVII cells. As in the experiment with OFU001, exponentially growing 250 SCCVII cells were plated onto dishes. After 7.5 h, the medium was replaced with MEM containing 2.5, 5, or 10 μM 5-FU and 10% serum, and 1, 3, 6, 12, or 24 h later, the drug-containing medium was removed, then the cultures were washed once, and replenished with fresh MEM.

Since OFU001 possesses electron-capturing capacity, the electron-affinic radiosensitizing activity of OFU001 was also investigated. Following published methods,¹³⁾ SCCVII cells were treated with 1 mM OFU001 in sealed test tubes 40 min prior to and during irradiation under hypoxic conditions. The concentration of 1 mM has been most often used to test radiosensitizers in our laboratory.^{11,13)} Immediately after irradiation with 4 MV X rays, the drug was removed by centrifugation and the cells were replated. To investigate aerobic radiosensitization, SCCVII cells plated 7.5 h beforehand were treated in dishes with 1 mM OFU001 40 min prior to and during

irradiation. After irradiation, the drug-containing medium was removed, and the cultures were washed, and replenished with fresh medium.

RESULTS

Radiation chemistry Fig. 2 shows dose-response curves for the decomposition of OFU001 and the release of 5-FU upon irradiation of a solution of 0.1 mM OFU001 in phosphate buffer under aerobic or hypoxic conditions. Both reactions were radiation-dose dependent, and the dose-response curves could be fitted with straight lines by linear regression (all $r > 0.988$, $P < 0.05$). While the decomposition of OFU001 was slightly more efficient with hypoxic radiolysis than with aerobic radiolysis, the release of 5-FU was greatly enhanced under hypoxic conditions. The G -value (mol number of molecules that are decomposed or produced by 1 J of absorbed radiation energy) for decomposition of OFU001 was 3.4 (95% confidence interval, CI, 3.1 to 3.8) $\times 10^{-7}$ mol/J under hypoxic conditions and 2.5

(CI, 2.1 to 2.9) $\times 10^{-7}$ mol/J under aerobic conditions, while the G -values for release of 5-FU were 1.9 (CI, 1.8 to 2.0) $\times 10^{-7}$ mol/J and 1.0 (CI, 0.95 to 1.04) $\times 10^{-8}$ mol/J, respectively. Thus, 55% of decomposed OFU001 was converted to 5-FU upon irradiation under hypoxic conditions. The release of 5-FU from OFU001 (0.1 mM) in MEM occurred with the same efficiency as in the phosphate buffer, with a G -value for 5-FU release of 1.9 (CI, 1.7 to 2.2) $\times 10^{-7}$ mol/J. The release of 5-FU was substantially identical (G -value: 1.9 [CI, 1.8 to 2.0] $\times 10^{-7}$ mol/J) even when the concentration of OFU001 was increased to 1 mM.

The metabolites of OFU001 other than 5-FU produced by aerobic and hypoxic irradiation are now under investigation. Following aerobic irradiation, there were three major peaks on the HPLC chart. One of them has been

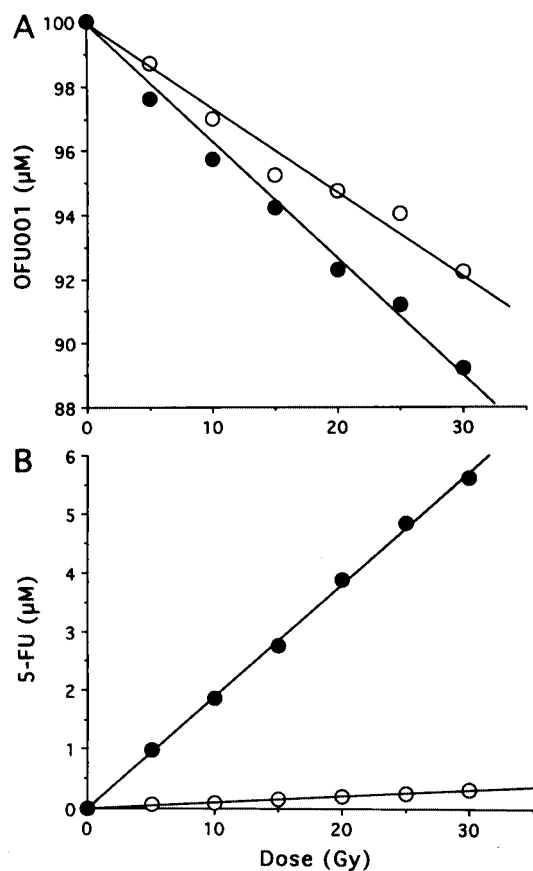


Fig. 2. Decomposition of OFU001 (A) and release of 5-FU (B) in phosphate buffer after aerobic (○) or hypoxic (●) irradiation.

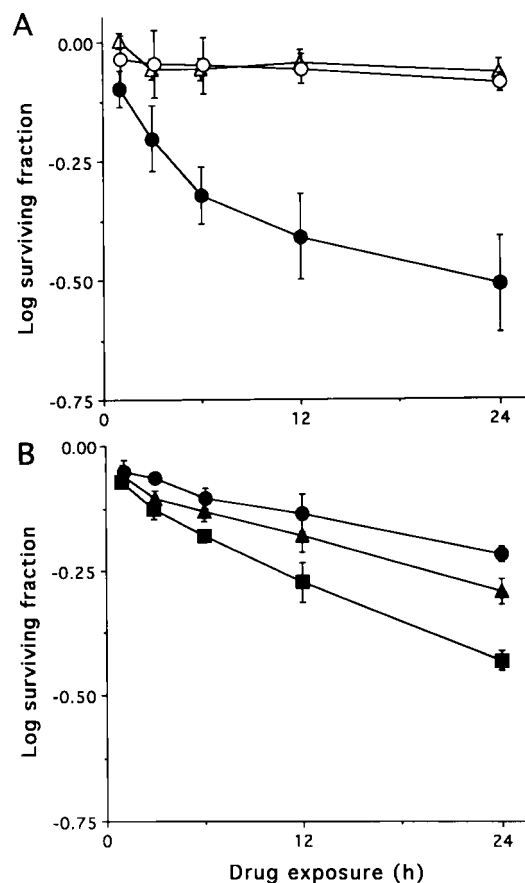


Fig. 3. Cytotoxic effect of OFU001 against SCCVII cells. A, OFU001-containing medium was sham-irradiated (Δ), aerobically irradiated with 30 Gy (\circ) or hypoxically irradiated at 30 Gy (\bullet), and then added to SCCVII cells. B, OFU001-containing medium was irradiated under hypoxic conditions with lower doses. \bullet 7.5 Gy, \blacktriangle 15 Gy, \blacksquare 22.5 Gy. Error bars represent SE of 3–4 experiments.

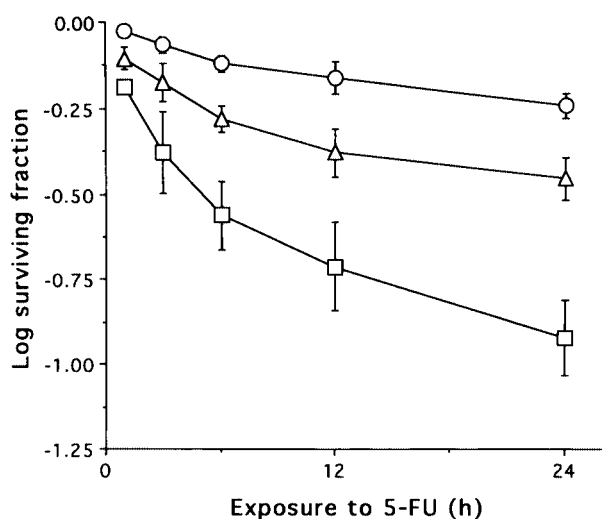


Fig. 4. Cytotoxic effect of different concentrations of 5-FU against SCCVII cells. ○ 2.5 μM , Δ 5 μM , \square 10 μM . Error bars represent SE of 3–4 experiments.

shown to represent 1-(2'-oxopropyl)-5-hydroxyuracil, and the identity of the other two major metabolites is now being determined (data to be published). Following hypoxic irradiation, however, there were no major peaks other than those for OFU001 and 5-FU on the HPLC chart.

In vitro effects Fig. 3 compares the cytotoxic effects of irradiated and unirradiated OFU001. OFU001 aerobically irradiated with 30 Gy or unirradiated OFU001 showed minimal cytotoxicity, and after 24 h of contact with them, only 15–19% of SCCVII cells were killed. On the other hand, OFU001 hypoxically irradiated with 30 Gy showed much higher cytotoxicity; the cell survival was 29% (95% CI, 24 to 39%) after 24 h of contact. This cytotoxic effect was clearly dependent on the drug contact time and the radiation dose.

Fig. 4 shows the cytotoxic effect of 5-FU on SCCVII cells. The effect was dependent on the concentration and contact time. The effects of 5-FU at 2.5 and 5 μM were nearly equivalent to those of OFU001 irradiated hypoxically with 15 and 30 Gy, respectively. From Fig. 2, the concentration of 5-FU released from OFU001 is estimated to be 2.8 μM at 15 Gy of hypoxic irradiation and 5.5 μM at 30 Gy. Since 10% serum was added to the medium containing OFU001 after irradiation, the final concentration of 5-FU in the medium should be approximately 2.5 μM after 15 Gy and 5 μM after 30 Gy. Thus, the cytotoxic effects of hypoxically irradiated OFU001 agreed well with those of 5-FU at the corresponding concentrations.

Fig. 5 shows the absence of a radiosensitizing effect of OFU001. When OFU001 was removed immediately after

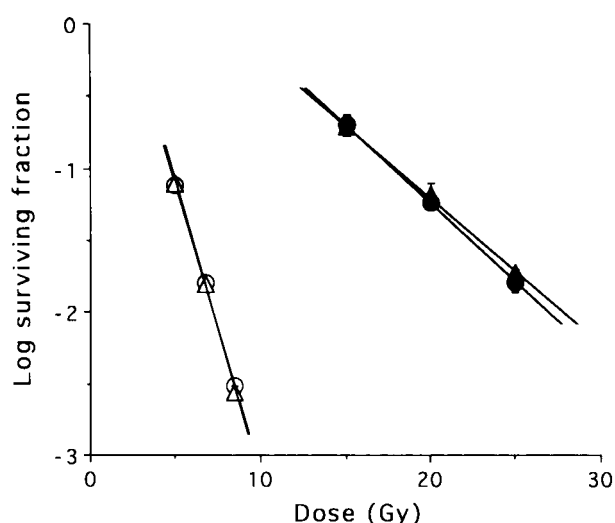


Fig. 5. Absence of radiosensitization by OFU001 (1 mM). SCCVII cells were irradiated with OFU001, which was removed immediately after irradiation. ○ aerobic, Δ aerobic+OFU001 1 mM, ● hypoxic, \blacktriangle hypoxic+OFU001 1 mM. Error bars represent SE of 3 experiments.

irradiation, the compound (1 mM) had no effect under either aerobic or hypoxic conditions.

DISCUSSION

Among various types of antitumor prodrugs, OFU001, which is activated by hypoxic irradiation, clearly represents a novel class. Regarding the mechanism of activation shown in Fig. 1, attempts are being made in our laboratory to confirm the formation of the anion radical intermediates using pulse radiolysis. In this regard, Mori *et al.*¹⁴ have detected the transient absorption of carbonyl π^* anion radicals following hypoxic irradiation of the N(1)-C(5) linked 5-FU dimer in solution in phosphate buffer. The incorporation of the radiochemically generated hydrated electron into the carbonyl group of electron-affinic compounds was previously reported by Adams and Dewey.¹⁵ The contribution of the σ^* anion radical that is in resonance with the π^* anion radical to the reductive dissociation of the C-Cl bond has been discussed by Santiago *et al.*¹⁶ Therefore, the reaction process shown in Fig. 1 is a combination of two reactions that were previously reported independently. Hydrated electrons are abundantly produced during hypoxic irradiation, but upon aerobic irradiation, the hydrated electrons are captured by electron-affinic oxygen to form reactive superoxide anion radicals ($\text{O}_2^{\cdot-}$). Therefore, this type of activation of OFU001 is essentially specific to irradiation under hypoxic conditions. The 2-oxo structure of the side chains at the N(1)-position of 5-FU

has been proven to play a crucial role in the release of 5-FU.⁹⁾ In contrast to OFU001, an analogue of OFU001 bearing a 1-propyl substituent without the 2-oxo function released no 5-FU upon irradiation under hypoxic conditions.⁹⁾ Recently, Wilson *et al.*¹⁷⁾ reported prodrugs of mechlorethamine which are activated by metabolic or radiolytic nitroreduction under hypoxic conditions. Those compounds are also hypoxia-specific, but their mechanism of activation is different from that of our compound.

Among many prodrugs that we have investigated, OFU001 possesses the simplest side chain and has one of the highest efficiencies of releasing 5-FU. The *G*-value of 1.9×10^{-7} mol/J for 5-FU release was much higher than that of the previously reported 5-FU dimer (7.5×10^{-8} mol/J).⁸⁾ Therefore, we investigated the efficacy of OFU001 in more detail. It exhibited good characteristics as a prodrug in *in vitro* experiments. OFU001 itself had very low cytotoxicity against SCCVII cells. In phosphate buffer, OFU001 was converted 19 times more efficiently into 5-FU following hypoxic than aerobic irradiation. This *G*-value ratio (hypoxia to air) was much higher than that (2.4) for the 5-FU dimer.⁸⁾ In accord with the radiation chemical reactivity, OFU001 had very low cytotoxicity when irradiated aerobically, but it showed definite effects when irradiated hypoxically. The cytotoxic effects of a given concentration of hypoxically irradiated OFU001 against SCCVII cells were similar to those of 5-FU at a concentration equivalent to that released by the OFU001. This observation suggests that the other byproducts derived from radiolysis of OFU001 are not cytotoxic.

This process of radiation activation is expected to work also *in vivo*, and we have started *in vivo* evaluation of this compound. In a preliminary study, we have observed release of 5-FU in *in vivo* tumors following intraperitoneal injection of OFU001 and irradiation of air-breathing mice. The amount of 5-FU release was similar to that expected from the *in vitro* experiments (Shibamoto *et al.*, data to be published). When $5 \mu\text{M}$ 5-FU is released within hypoxic tumor cells, some degree of *in vivo* effect may be expected, judging from the clinically achievable 5-FU concentrations in tumors and the effect of 5-FU at those

dosages.^{18,19)} To obtain this concentration of 5-FU, however, radiation doses of 25–30 Gy are necessary. These doses are used in clinics for intraoperative radiotherapy and radiosurgery, which are rapidly being introduced worldwide. So, OFU001 may be useful in clinics when combined with these treatment modalities, but more efficient compounds would be more suitable for clinical application.

We have therefore surveyed many other compounds having various 2-oxo side chains at the N(1)-position of 5-FU. Some compounds showed slightly, but not greatly, higher efficiency of release of 5-FU than OFU001, and at present it seems difficult to develop prodrugs of 5-FU which are more suitable for clinical application using this idea. However, we have confirmed that the concept of hypoxic radiation activation is applicable to other antitumor and cytotoxic agents (Nishimoto *et al.*, unpublished data). The 2-oxo side-chain structure seems to be important for developing radiation-activated prodrugs of other anticancer agents, as well. We have already synthesized several prodrugs of 5-fluoro-2'-deoxyuridine (FdUrd) having this type of side chain at their N(3)-position and are now investigating these compounds. FdUrd is one order of magnitude more efficient than 5-FU, in terms of the IC_{50} (molar concentration causing 50% growth inhibition) values in most cell lines.²⁰⁾ Thus, FdUrd-releasing prodrugs are expected to work better than 5-FU-releasing prodrugs, including OFU001. Furthermore, we are investigating various other anticancer drugs, such as mitomycin C, that are more efficient on a molar concentration basis than 5-FU and FdUrd and are capable of carrying 2-oxoalkyl side chains.

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