**FF-10502, an Antimetabolite with Novel Activity on Dormant Cells, Is Superior to Gemcitabine for Targeting Pancreatic Cancer Cells**

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**ABSTRACT**

In this paper, we report that 1-(2-deoxy-2-fluoro-4-thio-β-D-arabinofuranosyl) cytosine (FF-10502), a pyrimidine nucleoside antimetabolite with a chemical structure similar to gemcitabine, shows beneficial anticancer activity via a novel mechanism of action on dormant cells. The growth inhibition of pancreatic cancer cells by FF-10502 (IC50, 60–330 nM) was moderately weaker than that by gemcitabine in vitro. In contrast, an in vivo orthotopic implantation model in mice with established human pancreatic cancer cell line, SUIT-2, revealed no mortality with FF-10502 intravenous treatment, which was related to regression of implanted tumor and little metastasis, whereas 75% of the mice treated with gemcitabine died by day 128. Two in vivo patient-derived xenograft models with gemcitabine-resistant pancreatic cancer cells also demonstrated complete tumor growth suppression with FF-10502, but only partial inhibition with gemcitabine.

We also investigated the mechanism of action of FF-10502 by using dormant cancer cells, which are reportedly involved in the development of resistance to chemotherapy. In vitro serum starvation–induced dormant SUIT-2 cells developed resistance to gemcitabine even in combination with DNA damage inducers (DDIs; H2O2, cisplatin, and temozolomide). Interestingly, FF-10502 in combination with DDIs significantly induced concentration-dependent cell death in accordance with enhanced DNA damage. FF-10502 was far more potent than gemcitabine in inhibiting DNA polymerase β, which may explain the difference in dormant cell injury, although further investigations for direct evidences are necessary. In conclusion, our study demonstrated the beneficial antitumor effects of FF-10502 in clinically relevant in vivo models, and suggests the importance of preventing DNA repair unlike gemcitabine.

**Introduction**

Gemcitabine, an antitumor chemotherapy drug classified as a pyrimidine nucleoside antimetabolite, was approved by the US Food and Drug Administration in 1996 primarily as a result of an enhanced clinical benefit response. The current standard of care for patients with advanced or metastatic pancreatic cancer is gemcitabine-based chemotherapy. However, the antitumor effect of gemcitabine is modest, with a partial response rate of 5.4% and median survival time 1.3 months longer than that with 5-fluouracil (5-FU; 4.2 months) (Burris et al., 1997). Following the development of combination therapy with nab-paclitaxel, median survival time was extended by 2.1 months compared with treatment with gemcitabine alone (6.6 months) (Goldstein et al., 2015). Another combination chemotherapy regimen consisting of oxaliplatin, irinotecan, 5-FU, and leucovorin (FOLFIRINOX) also extended the survival, and the median overall survival was 11.1 months in the FOLFIRINOX group as compared with 6.8 months in the gemcitabine group (Conroy et al., 2011). Although these new regimens represent significant improvements, the 5-year survival rate for patients with pancreatic cancer remains only 7%, the lowest among all types of cancer (Siegel et al., 2015). It is especially concerning that a poor prognosis is associated with poor response to chemotherapy and tumor recurrence. Specifically,
human pancreatic cancer can acquire resistance to conventional chemotherapeutics, including paclitaxel, 5-FU, cisplatin, and gemcitabine (Arumugam et al., 2009). One such mechanism of drug resistance in pancreatic cancer is the enrichment of dormant cell populations (cancer stem cells and/or nonproliferating, quiescent cells). Stanton et al. (2003) reported that 28% ± 15% of pancreatic cancer cells were positive for nuclear Ki67 in a study of 33 pancreatic adenocarcinomas, suggesting that over 70% of pancreatic tumor cells are dormant. Those dormant cells—including cancer stem cells—possess “robustness,” a property that encompasses the characteristics of a slow cell cycle, resistance to oxidative stress, and a rapid response to DNA damage, all of which contribute to the development of therapeutic resistance (Yoshida and Saya, 2016). Thus, to advance the treatment of pancreatic cancer, it is necessary to develop novel approaches that target these dormant cells.

FF-10502 [1-(2-deoxy-2-fluoro-4-thio-D-arabinofuranosyl)cytosine], formerly known as 4’-thio-FAC, was discovered by the Yamasa Corporation in Japan and developed as an anticancer chemotherapy drug (Miura et al., 1998). FF-10502 is classified as a pyrimidine nucleoside antimetabolite and has a chemical structure similar to gemcitabine (Fig. 1). FF-10502 has a sulfur atom in its sugar ring instead of oxygen, and has one fluorine atom compared with gemcitabine’s two fluorine atoms at the 2’ position in the sugar moiety (Miura et al., 1999). The antiproliferative and cytotoxic effects of FF-10502 have been tested in vitro on many types of solid tumors, including pancreas, lung, stomach, colon, breast, ovary, bladder, melanoma, osteosarcoma, and head and neck cancers (Miura et al., 1998, 1999; Zajchowski et al., 2005). It is noteworthy that FF-10502 has shown superior efficacy compared with gemcitabine in all in vivo studies reported thus far (Miura et al., 1998, 1999, 2002; Zajchowski et al., 2005).

Previous reports have revealed elements of the mechanism of action of FF-10502. After its uptake into cells, FF-10502 is metabolized to FF-10502-triphosphate (FF-10502TP), an active metabolite of FF-10502 similar to gemcitabine-triphosphate (gemTP) (Heinemann et al., 1988). Compared with gemTP, the inhibitory activity of FF-10502TP on DNA polymerase α (polα) and polymerase β (polβ) was approximately 1000 and 100 times higher, respectively. Therefore, it was hypothesized that the efficacy of FF-10502TP2 can be attributed to the inhibition of polα (Miura et al., 2001; Miura and Izuta, 2004). However, this potent inhibition of polα is not consistent with tumor cell growth inhibition in vitro, as the IC50 values for FF-10502 in tumor cell growth assays are generally higher than those of gemcitabine. This does not explain why FF-10502 has shown efficacy superior to gemcitabine in animal models of tumors in vivo.

To explain the discrepancy between the in vitro and in vivo efficacy of FF-10502 and gemcitabine, we further examined the pharmacological profile of FF-10502, with an objective to elucidate the mechanisms involved. This study focused on pancreatic cancer, because dormancy of this type of tumor has been suggested to be the key factor in the differences in efficacy. The effect of FF-10502 in relation to the inhibition of DNA repair after exposure of dormant cells with chemotherapy resistance to DNA damage inducers (DDIs) and the importance of the novel mechanism involved in the effect are discussed.

Figure 1. Chemical structures of FF-10502 and gemcitabine.

Materials and Methods

Chemicals and Reagents. FF-10502 methanesulfonate (FF-10502-01) and FF-10502TP were synthesized and provided by FUJIFILM Corporation (Tokyo, Japan). For simplicity, FF-10502 methanesulfonate is referred to as “FF-10502” in this study. Gemcitabine hydrochloride was purchased from Teva Pharmaceutical Industries (Netanya, Israel) and is designated as “gemcitabine” in this study. The formulation used in the in vivo study represents the free base for both compounds. FF-10502 and gemcitabine were dissolved in phosphate-buffered saline (PBS) for in vitro studies, in dimethylsulfoxide (DMSO) for a combination study with DDIs, or in saline for in vivo studies. GemTP was purchased from Jena Bioscience (Jena, Germany). Three DDIs (H2O2, cisplatin, and temozolomide) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan); Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and LKT Laboratories, Inc. (St. Paul, MN), respectively. All DDIs were dissolved in culture medium.

Cell Lines. BxPC-3 cells and Capan-1 cells were obtained from the American Type Culture Collection (Manassas, VA), and SUIT-2 cells and Mia PaCa-2 cells were obtained from the Japanese Collection of Research Bioreources (Osaka, Japan). BxPC-3 and SUIT-2 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. Capan-1 and Mia PaCa-2 cells were cultured in Iscove’s modified Dulbecco’s medium (Thermo Fisher Scientific) with 20% heat-inactivated FBS, and minimum essential medium with 10% heat-inactivated FBS plus 1% nonessential amino acids, respectively. All culture media contained 100 units/ml penicillin and 100 µg/ml streptomycin. The cultures were incubated in a CO2 incubator at 37°C with 5% CO2 in a humidified atmosphere. All cells were subcultured every 3–4 days.

Cell Growth Inhibition Assay. Cells were seeded at 1000 cells/well (BxPC-3, SUIT-2, and Mia PaCa-2) or 3000 cells/well (Capan-1) into 96-well culture plates. After a 24-hour culture, FF-10502, gemcitabine, or PBS alone (control) was added to the wells. The cells were incubated for approximately 72 hours. Cell growth inhibition was evaluated using a CellTiter-Glo Luminescent Cell Viability kit (Promega, Madison, WI). Luminescence was measured using an EnVision plate reader (PerkinElmer, Waltham, MA). The IC50 value of the test substance against cell growth was calculated using Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA).

Subcutaneous Implantation Model with Human Pancreatic Cancer Cell Line Capan-1. The experiment was performed in accordance with the Guide for the Care and Use of Laboratory Animals and was approved by the FUJIFILM animal experiment committee. Five-week-old female nude mice (BALB/c-nu/nu) were purchased from CLEA Japan SLC Inc. (Tokyo, Japan). A Capan-1 cell suspension of 1 × 10⁶ cells/ml was prepared with serum-free medium; 100 µl of the
cell suspension was subcutaneously injected into the right flank region of each animal. Nine days after implantation, the mice were randomized into nine groups (10 mice/group), and 100 µl/kg of saline (vehicle solution), gemicitabine, or FF-10502 was administered by tail vein injection once weekly for 4 weeks. Tumor diameter and body weight were measured twice weekly. For calculation of tumor volume, both long and short diameters (mm) were measured by a Vernier caliper (Mitutoyo, Kawasaki, Japan). The formula for calculating tumor volume was as follows: tumor volume (mm$^3$) = long diameter (mm) $\times$ short diameter (mm) $\times$ short diameter (mm) $\times$ 0.5.

**Orthotopic Implantation Model with Human Pancreatic Cancer Cell Line SUIT-2.** The experiment was performed in accordance with the Guide for the Care and Use of Laboratory Animals and was approved by the FUJIFILM animal experiment committee. Five-week-old female nude mice (BALB/c-nu-nu) were purchased from CLEA Japan SLC Inc. A SUIT-2 cell suspension of 1 $\times$ 10$^7$ cells/ml was prepared in serum-free medium, and 10 µl of the cell suspension was injected into the pancreas of each animal under isoflurane anesthesia. After confirmation that there was no hemorrhage at the site of implantation, the abdominal wall incision was closed with absorbable sutures, and the skin was clamped. Seven days after implantation, at which metastases were already observed (Higuchi et al., 2018), the mice were randomized into five groups (20 mice/group), and 100 µl/kg of saline (vehicle solution), gemicitabine, or FF-10502 was administered by tail vein injection once weekly for 18 weeks.

Antitumor activity of the orthotopic implantation model was evaluated by event-free survival time, defined as the day from SUIT-2 cell injection until death or morbiditiy (e.g., marked decrease in body weight, hypothermia, or other conditions requiring euthanasia). Histopathological evaluation was also performed for various tissues of surviving animals stained with hematoxylin and eosin.

**Patient-Derived Xenograft Model.** Animal welfare for this experiment complied with the US Department of Agriculture’s Animal Welfare Act (9 Code of Federal Regulations Parts 1, 2, and 3), as applicable. Female NOD-SCID mice were purchased from Harlan Laboratories (Indianapolis, IN). Two patient-derived pancreatic cancer cells (PA5364 and PA5365) were used. These are adenocarcinoma and carcinoma cells derived from ascites of a 78-year-old female and the omentum of a 54-year-old female, respectively. PA5364 shows high resistance to 5-FU and carbustine, intermediate resistance to gemcitabine and mitomycin C, and low resistance to 5-FU, docetaxel, and SN38 (an active metabolite of irinotecan). The cell suspensions of 2.5 $\times$ 10$^6$ cells/ml (PA5364) or 1.2 $\times$ 10$^6$ cells/ml (PA5365) were prepared with PBS and mixed with an equal volume of Cultrex extracellular matrix (Trevigen, Gaithersburg, MD), and 200 µl of the cell suspension in Cultrex extracellular matrix was injected subcutaneously into the rear flank under isoflurane anesthesia. The animals were randomized into three groups (10 mice/group) when the average tumor size reached approximately 200 mm$^3$. Vehicle (saline), gemicitabine, or FF-10502 was administered by tail vein injection once weekly for 4 weeks, followed by 4 weeks of observation. Animals were monitored weekly for palpable tumors and any changes in appearance or behavior. Once tumors were palpable, they were measured using calipers. For calculation of tumor volume, both long and short diameters (mm) were measured by Vernier caliper (Mitutoyo). The formula for calculating tumor volume was as follows: tumor volume (mm$^3$) = long diameter (mm) $\times$ short diameter (mm) $\times$ short diameter (mm) $\times$ 0.5.

**Inhibitory Activity against Polo and Polb.** The inhibitory activity of FF-10502TP and gemTP for polo was measured by a DNA synthesis assay in vitro (Podust et al., 1989). Purified human polo (EuRx, Gdansk, Poland) and FF-10502TP or gemTP were incubated for 30 minutes at 37°C in 50 µl of reaction mixture [60 mM Tris-HCl (pH 8.0), 5.0 mM magnesium acetate, 0.3 mg/ml bovine serum albumin; 1.0 mM dithiothreitol; 0.1 mM spermine; 20 µM each dCTP, dGTP, and dATP; 5 µM [H-methyl]dTTP; and 20 µg of activated calf thymus DNA]. Likewise, purified human polb (EuRx) and FF-10502TP or gemTP were incubated for 15 minutes at 37°C in 50 µl of reaction mixture [50 mM Tris-HCl (pH 8.7), 10.0 mM MgCl$_2$, 0.4 mg/ml bovine serum albumin; 1.0 mM dithiothreitol; 100 mM KCl; 15% glycerol; 50 µM each dCTP, dGTP, and dATP; 5 µM [H-methyl]dTTP; and 10 µg of activated calf thymus DNA]. After incubation, 20 µl of the reaction solution was passed through Diethylaminoethyl-cellulose paper, and the membrane was washed with 5% Na$_2$HPO$_4$ (1 ml), Milli-Q water (1 ml), ethanol (1 ml $\times$ 2), and diethyl ether (1 ml). The DEAE-cellulose paper was transferred into a scintillation vial and 5 ml of scintillation fluid, and PICO-FLUOR PLUS (PerkinElmer) was added; radioactivity was measured with a liquid scintillation counter (PerkinElmer).

The inhibition rate (individual value) was calculated as 100 – response ratio (formula shown here). In addition, the mean inhibition rate of duplicate samples was calculated:

$$\text{response ratio} = \frac{\text{B} - \text{N}}{\text{B} - \text{N}_0} \times 100\%$$

where B = radioactivity of the sample measuring inhibitory activity (individual value), B$_0$ = radioactivity of the sample measuring total activity (mean value), and N = radioactivity of the sample measuring nonspecific activity (mean value). For data processing, Microsoft Excel 2003 (Microsoft Corporation) was used.

**Inhibitory Activity of DNA Synthesis in a SUIT-2 Orthotopic Implantation Tumor Model.** Mice with SUIT-2 orthotopic implantation were prepared in accordance with the procedure described earlier. On day 18, vehicle (saline), gemicitabine, or FF-10502 was administered by tail vein injection (100 µg/kg). The tumors were harvested 4, 24, 48, and 72 hours after drug administration. Mice were injected intraperitoneally with 100–200 µg of 5-ethyl2-deoxyuridine (EdU; Bacelick GmbH, Neuried, Germany) in PBS 4 hours before tumor harvest (Salic and Mitchison, 2008). Pieces of the tumor were formalin-fixed, embedded in paraffin, and sectioned. After paraffin removal, sections on glass slides were stained with 10 µM Alexa568-azide (Thermo Fisher Scientific) for 30 minutes at room temperature. Sections were counterstained with Hoechst 33342 (Thermo Fisher Scientific) and mounted for fluorescence microscopy (CQ1; Yokogawa Electric Corporation, Tokyo, Japan) to quantify the total cell number and DNA synthesizing cell number. The rate of EdU-incorporated cells was calculated as follows:

$$\text{EdU-incorporated cells} = \frac{N_E}{N_H} \times 100\%$$

where N$_E$ = number of EdU-incorporated cells (individual value), and N$_H$ = number of Hoechst-stained cells (individual value). For data processing, Microsoft Excel 2003 (Microsoft Corporation) was used.

**SUIT-2 Dormant Cell Model.** SUIT-2 cells were seeded at 15,000 cells/well into 96-well culture plates (CellBIND 96 well clear flat bottom; Corning, Corning, NY) with medium containing 10% FBS. After a 24-hour culture, the cells were washed twice with 150 µl of serum-free medium and then cultured in serum-free medium for 72 hours. FF-10502, gemicitabine, or 0.1% DMSO alone (control) was added to the wells. H$_2$O$_2$ (final concentration of 300 µM), temozolomide (final concentration of 200 µM), or cisplatin (final concentration of 5 µM) was also added for combination treatment. The cells were incubated in a CO$_2$ incubator at 37°C with 5% CO$_2$ in a humidified atmosphere for approximately 72 hours, and cell growth inhibition was evaluated using the CellTitrer-Glo Luminescent Cell Viability Assay Kit (Promega). Luminescence was measured using an EnVision plate reader (PerkinElmer).

**Comet Assay in SUIT-2 Dormant Cell Model.** The SUIT-2 cell culture and test article treatments were performed according to the same procedure described earlier, with the exception that SUIT-2 cells were seeded at 100,000 cells/well in 2-well culture plates and combined with DDIs and FF-10502 or gemicitabine for 4 or 24 hours. After treatment, the cells were detached and resuspended in PBS at over 100,000 cells/ml. The cell suspension was mixed with Comet LMagarose (Trevigen) and placed onto slides (Matsunami adhesive silane-coated slide, 5 holes; Matsunami Glass Ind., Ltd., Osaka,
Japan). Cells on the slides were lysed with lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 10% DMSO, and 1% Triton X-100, pH 10) at 4°C for at least 30 minutes, and slides were immersed in electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13) for 20 minutes. Electrophoresis was then carried out at 21 V/cm for 30 minutes. The slides were washed twice with distilled water and immersed in 70% ethanol, then dried and stained with SYBR Gold (Thermo Fisher Scientific). Fluorescence microscopic images were scored quantitatively by an image analyzer system (Comet Assay IV, version 4.2; Perceptive Instruments Ltd., Edmunds, UK). Percentage of fluorescence intensity in the tail DNA region per whole DNA is expressed as % tail DNA. The experiment was performed in duplicate, and 60 comet cells/well were analyzed; the mean % tail DNA was calculated using the Comet Assay Spreadsheet Generator, version 1.3.1 (Perceptive Instruments Ltd.).

Statistical Analysis. Mouse event-free survival was graphically represented using Kaplan-Meier analysis, and was analyzed between groups with the log-rank test using SAS 9.2 (SAS Institute Japan Ltd., Tokyo, Japan) and the interlocking system Exsus version 7.7.1 (CAC EXICARE Corporation, Tokyo, Japan). The patient-derived xenograft (PDX) model was analyzed by one-way analysis of variance and Tukey’s test using the GraphPad Prism 5.04 software (GraphPad Software, Inc., La Jolla, CA). In comparison testing, the significance level was set at 5%.

Results

Antitumor Activity of FF-10502 In Vitro and In Vivo with Human Pancreatic Cancer Cell Lines. The inhibitory activities of gemcitabine and FF-10502 in four human pancreatic cancer cell lines (BxPC-3, SUIT-2, Capan-1, and MIA PaCa-2) were evaluated in vitro (Table 1). The average IC_{50} values (nanomolars per liter) of gemcitabine in these cell lines were 17.7, 3.7, 22.4, and 27.5, respectively, and those of FF-10502 were 59.9, 39.6, 68.2, and 331.4, respectively. These results were consistent with those reported in previous studies (Miura et al., 1999; Zajchowski et al., 2005).

The antitumor effect of FF-10502 was evaluated in a mouse xenograft model with the subcutaneously implanted human pancreatic cancer cell line Capan-1. Intravenous administrations of FF-10502 or gemcitabine at 120, 240, 360, and 480 mg/kg once weekly suppressed tumor growth in a dose-dependent manner (Fig. 2, A and B). The maximum decrease in body weight in FF-10502- and gemcitabine-treated mice was 14.5% at 360 mg/kg and 28.3% at 480 mg/kg, respectively. No deaths were observed in either treatment group.

The antitumor effect of FF-10502 was further evaluated in a mouse model of orthotopic implantation with the human pancreatic cancer cell line SUIT-2. The SUIT-2 cell line is derived from liver metastasis of pancreatic cancer patients with Kras and TP53 gene mutations; produces at least two tumor markers, carcinoembryonic antigen and carbohydrate antigen 19-9; and has been widely used since its establishment (Iwamura et al., 1987, 1992; Moore et al., 2001). The SUIT-2 cells can survive in mice after orthotopic implantation, which is known to reproduce the pattern of local tumor growth and distant metastasis observed in human pancreatic cancer (Shono et al., 2001; Higuchi et al., 2018). The first weekly intravenous administration of each dosing solution (20 mice/group) was performed 7 days after implantation, the same day that the implanted SUIT-2 cells began to metastasize to the mesentery and spleen. Treatments continued until day 128. Vehicle-treated mice started to die at day 19; their survival rate at day 128 (the final day of the study) after tumor implantation was 5%, and median survival was 54.5 days (Fig. 2C; Table 2). Gemcitabine treatment showed a dose-dependent and statistically significant prolongation of mouse survival compared with that of vehicle-treated mice. The survival rate at day 128 was 25% at the 240-mg/kg dose and 75% at the 480-mg/kg dose (Table 2). In contrast, all mice treated with 240 or 480 mg/kg of FF-10502 survived until the end of the study, yielding a survival rate of 100% at both doses. The effects were statistically significant compared with not only the vehicle-treated group but also the dose-matched gemcitabine-treated group.

A histopathological assessment of five surviving mice in each group revealed further contrast between gemcitabine and FF-10502 treatments (Fig. 2D). Increased sizes of implanted tumors at the pancreas were observed in all gemcitabine-treated mice at 240 and 480 mg/kg. In contrast, all mice treated with FF-10502 at 240 mg/kg showed regressions of the implanted tumors, and three out of five of the FF-10502–treated mice at 480 mg/kg showed no implanted tumors. Metastases at the mesentery were observed in all gemcitabine-treated mice in the 240-mg/kg group, and in two out of five of the 480-mg/kg group, whereas metastases were observed after FF-10502 treatment at the same doses in only one out of five mice in each group. Liver metastases were also observed in five out of five and two out of five of the gemcitabine-treated mice at 240 and 480 mg/kg, respectively. However, liver metastases were observed in only one out of five and zero out of five of the FF-10502–treated mice at 240 and 480 mg/kg, respectively.

Antitumor Activity of FF-10502 in Pancreatic Patient-Derived Cancer Cells In Vivo. We next evaluated the antitumor effects of gemcitabine and FF-10502 in a PDX model in vivo, in which two lines of gemcitabine-resistant, patient-derived pancreatic tumor cells were used. Four intravenous weekly treatments of gemcitabine or FF-10502 were initiated when the average tumor size reached 200 mm^3. The tumors receiving gemcitabine treatment showed slow growth, which turned into rapid growth after the last drug administration on day 22. In contrast, the FF-10502–treated groups showed statistically significant tumor shrinkage at 240 mg/kg, and the effect continued even after the termination of treatment (Fig. 3).

Mechanism of Action of FF-10502. Although the principal mechanisms of FF-10502 antitumor activity remain to be determined, some studies have indicated a potential role of DNA pola inhibition, an enzyme involved in initiation of DNA replication (Miura et al., 2001; Miura and Izuta, 2004). Miura

| Cell line     | FF-10502 | Gemcitabine |
|--------------|----------|-------------|
| BxPC-3       | 59.9 ± 11.5 | 17.7 ± 4.9  |
| SUIT-2       | 39.6 ± 0.7  | 3.7 ± 0.1   |
| Capan-1      | 68.2 ± 2.7  | 22.4 ± 1.6  |
| MIA PaCa-2   | 331.4 ± 233.8 | 27.5 ± 9.8 |

| Cell growth inhibitory activity of FF-10502 and gemcitabine in human pancreatic cancer cell lines | IC_{50} (nM) | Cell line     | FF-10502 | Gemcitabine |
|-------------------------------------------------|------------|--------------|----------|-------------|
|TABLE 1                                          |            |             |          |             |

Cells were cultured in the presence of FF-10502 or gemcitabine at concentrations from 0.1 nM to 10 μM for 72 hours. Cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay Kit. Mean IC_{50} values ± S.D. are presented for experiments performed at least three times.
et al. (2001) reported that the inhibitory activity of FF-10502TP for pol\(\alpha\) was approximately 1000 times more potent than that of gemTP. This was confirmed in our current study, as shown in Fig. 4A; the inhibitory activity of FF-10502TP against pol\(\alpha\) was more potent than that of gemTP, although the potency was 14-fold higher (IC\(_{50}\) was 23 \(\mu\)M for FF-10502TP and 547 \(\mu\)M for gemTP). Therefore, it was hypothesized that the antitumor activity of FF-10502TP is...
dependent on its inhibitory activity for polα; consequently, we evaluated DNA synthesis activity in tumors using a mouse model of orthotopically implanted SUIT-2 cells by measuring the percentage of EdU incorporated into DNA after FF-10502 or gemcitabine administration. Both FF-10502 and gemcitabine inhibited DNA synthesis from 4 to 48 hours, but DNA synthesis recovered by 72 hours. No significant differences were observed in the inhibitory activities for DNA synthesis between FF-10502 and gemcitabine (Fig. 4, C and D).

We next examined polβ, in light of a previous study that reported the potency of FF-10502TP against polβ to be 23 times higher than that of gemTP (Miura et al., 2001). This trend was confirmed in our current study, although gemTP did not reach 50% inhibition while the IC50 of FF-10502TP was 10 μM (Fig. 4B). Polβ is known to be a DNA-repairing enzyme that plays a key role in base excision repair (BER); therefore, DDIs causing DNA damage (repaired by BER) were combined with FF-10502 or gemcitabine to investigate whether polβ inhibitory activity contributes to its cytotoxic effect. To avoid antiproliferation effects by polα inhibition with FF-10502 or gemcitabine, we established a SUIT-2 dormant cell model that was induced by serum-free medium (Fig. 5A). SUIT-2 cells did not proliferate, and cell death was not evident by 72 hours in serum-free conditions. Individual treatment of gemcitabine or FF-10502, or DDIs alone, did not affect cell viability (Fig. 5B). Under these conditions, gemcitabine did not affect, or weakly affected, cell viability in combination with DDIs. However, FF-10502 induced significant cell death in combination with H2O2, cisplatin, or temozolomide (Fig. 5, C–E). The comet assay was applied to confirm DNA damage; the results clearly indicated that the combination of FF-10502 with DDIs caused synergistic DNA damage, whereas the combination of gemcitabine with DDIs yielded little response (Figs. 6 and 7; Table 3).

**Discussion**

In most of the previous studies, FF-10502 was evaluated in mice models of subcutaneously implanted pancreatic cancer cell lines in vivo and showed superior efficacies compared with gemcitabine (Miura et al., 1998, 1999, 2002; Zajchowski et al., 2005). We further evaluated FF-10502 in our study using more clinically relevant models in vivo, i.e., orthotopic implantation of human pancreatic cancer SUIT-2 cells and subcutaneous implantation of gemcitabine-resistant patient-derived cells. The SUIT-2 cell line has been widely used in vitro and in vivo since its establishment from metastasis in livers of human pancreatic patients. The in vivo orthotopic implantation model with SUIT-2 in mice has been validated and shows similarities to human pancreatic patients in terms of metastases at the peritoneum, diaphragm, liver, and lungs, and the partial response to gemcitabine (Tomioka et al., 2001; Higuchi et al., 2018). Our study using the model revealed that FF-10502–treated mice showed prominent inhibition of implanted tumors and little or no hepatic or intraperitoneal metastasis, whereas increased sizes of implanted tumors and metastases were observed in gemcitabine-treated mice (Fig. 2D). The results suggested that these histopathological observations were related to survival of all animals.
mice treated with FF-10502, unlike gemcitabine (Fig. 2C). The PDX models with pancreatic cancer cells from patients with resistance to gemcitabine also showed clear difference between FF-10502 and gemcitabine (Fig. 3). These are new findings showing the superior efficacy of FF-10502 to gemcitabine in those clinically relevant animal models of pancreatic cancer.

The growth-inhibitory activity of FF-10502 in vitro human pancreatic cancer cell lines (BxPC-3, SUIT-2, Capan-1, and MIA PaCa-2) was lower than that of gemcitabine (Table 1). However, the antitumor activity of FF-10502 in vivo was superior to that of gemcitabine, as demonstrated in mouse models of Capan-1 xenografts, SUIT-2 orthotopic implantation, and PDX with pancreatic cancer cells in vivo.

We hypothesized that the lower efficacy of gemcitabine in vitro is caused by the presence of slow growth or dormant cells. It is empirically known that implanted cells in vivo grow...
much slower than cultured cells in vitro. Amikura et al. (1995) reported that the doubling time of metastatic pancreatic tumor cells in the liver in vivo was approximately 15 times longer than their doubling time in culture. In this study, we demonstrated that 90% of implanted SUIT-2 cells did not proliferate (Fig. 4, C and D). Gemcitabine is not cytotoxic to cells that grow very slowly or acquire dormancy, since the primary target of gemcitabine is the DNA synthesis enzyme polα. For example, the potency of gemcitabine is approximately 100 times lower in a quiescent Capan-2 spheroid culture than in a proliferative monolayer culture (Dufau et al., 2012). In accordance with this prior report, we hypothesized that gemcitabine did not affect dormant cells cultured in serum-free medium (Fig. 5B). Accordingly, we hypothesized that the higher efficacy of FF-10502 observed in the in vivo model can be attributed to cytotoxicity against slow-growing or dormant cells, which emerge due to acquired heterogeneity in vivo, and that FF-10502 may have an additional unique mechanism of action that is distinct from the mechanism of gemcitabine.

In previous studies also conducted in mice, the pharmacokinetics of FF-10502 and gemcitabine were comparable, and FF-10502 showed a high degree of inhibitory activity against polα (Miura et al., 2001; Zajchowski et al., 2005). It was hypothesized, therefore, that the superior antitumor activity of FF-10502 was caused by high polα inhibitory activity.

Fig. 5. Cytotoxic effect of DDI treatment in combination with either FF-10502 or gemcitabine on pancreatic cancer dormant cells. (A) Study design of the SUIT-2 dormant cell model. After 3 days of culture in serum-free medium, cells were incubated with FF-10502 or gemcitabine at concentrations from 1 to 10 μM in the presence or absence of DDIs for 3 days. The viability of the remaining cells was evaluated by the CellTiter-Glo Luminescent Cell Viability Assay kit. Cell viability was plotted at each concentration of FF-10502 and gemcitabine in the absence of DNA damaging inducers (B) and the presence of 300 μM H₂O₂ (C), 5 μM cisplatin (D), and 200 μM temozolomide (E).
However, there was no significant difference in the inhibition of DNA synthesis between FF-10502 and gemcitabine in the SUIT-2 orthotopic implantation model (Fig. 4, C and D). These results suggest that inhibitory activity against polα cannot account for the pronounced antitumor activity of FF-10502 in vivo. Thus, we hypothesized that FF-10502 may have additional mechanisms of action.

Although a previous study suggested that FF-10502 did not affect the RNA transcription in the growing cell (Miura et al., 2001), a question was raised as to whether it would be the same in dormant cells. 5-FU is known as a DNA and RNA synthesis inhibitor, and we evaluated the cytotoxicity at 0–100 μM in our serum starvation assay in combination with 10 μM cisplatin. As a result, 5-FU did not affect the cisplatin-induced cytotoxicity even at the highest concentration (100 μM) (Supplemental Material). The results suggest that transcriptional inhibition is less likely as the mechanism of action in the enhanced cisplatin-induced cytotoxicity by FF-10502 in dormant cells.

We then focused on polβ inhibitory activity, as FF-10502 showed a much greater degree of inhibition against polβ than gemcitabine (Fig. 4B). The observed inhibition of polβ with FF-10502 was much more prominent in this study than in previous reports. The reason for this difference is not clear, except perhaps differing sources of polβ (calf thymus in the previous report and recombinant human protein in this study). Polβ, which is induced by H2O2 or temozolomide, is a principal DNA polymerase in BER and has been explored as a cancer therapeutic target (Lange et al., 2011; Kim and Wilson, 2012). In previous studies, small-molecule polβ inhibitors
enhanced the cytotoxicity of bleomycin or temozolomide in lung and colon cancers (Gao et al., 2008; Jaiswal et al., 2009), and interference in polβ expression increased sensitivity to oxaliplatin (Yang et al., 2010). Therefore, we further investigated the relationship between the inhibitory activity of FF-10502 against polβ and dormant cell viability using a SUIT-2 serum-starvation model. The cell viability of SUIT-2 dormant cells was not affected by treatment with H2O2 alone, but was dramatically reduced by combining H2O2 with FF-10502; however, this effect was not observed with gemicitabine (Fig. 5C). Similar results were obtained with temozolomide, which causes DNA damage that is repaired by BER (Fig. 5G). These results suggest that FF-10502 inhibits BER of dormant tumor cells and causes cell death in the presence of DDIs.

It is known that high amounts of reactive oxygen species are generated in tumors and cause DNA damage, such as base oxidation (Szatrowski and Nathan, 1991; Trachootham et al., 2006). Therefore, the high efficiencies of FF-10502 in vivo may be explained by endogenous oxidative DNA damage in tumors plus polβ inhibition by FF-10502.

DNA polymerase δ, ε, and κ, as well as polβ, are known to be involved in cisplatin-induced DNA damage (Ogi et al., 2010). The observation that FF-10502 also enhanced the cytotoxicity of cisplatin in dormant cells (Fig. 5D) further suggests that there are other target polymerases for FF-10502.

It may be argued that polβ inhibition causes deleterious effects because of the essential role it plays in BER, but there were no serious toxicities observed with FF-10502 treatment in our animal models, and a clinical trial of FF-10502 that is currently ongoing in the United States has demonstrated good tolerability in patients with solid tumors (Falchook et al., 2017).

Taken together, these results imply that polβ inhibition may play a role in the mechanism contributing to the difference in efficacy between FF-10502 and gemicitabine, although further experiments will be necessary to show direct evidences for the contribution of polβ inhibition as well as the involvement of other DNA polymerases.

Cancer stem cells (CSCs) are accepted as the cause of relapse and distant metastasis in cancer progression (Rasheed et al., 2011; Podberezin et al., 2013; Li and Li, 2014). SUIT-2 cells express CSC markers (CD133+ and CXCR4+) (Moriyama et al., 2010), and our study indicated that orthotopically implanted SUIT-2 cells metastasize aggressively and are resistant to gemicitabine. Despite repeated high-dose administration of gemicitabine (480 mg/kg), tumor progression was not prevented. In contrast, FF-10502 treatment resulted in no deaths at doses of both 240 and 480 mg/kg; FF-10502 also inhibited dissemination of tumor cells and reduced tumor cell growth at the inoculated site. These results suggest that FF-10502 displays a wide spectrum of antitumor activity that includes targeting rapidly growing and dormant cells (including CSCs); this new insight may encourage the exploration of novel research directions for cancer stem cell therapeutics.

The effect of FF-10502 on the tumor microenvironment such as interactions with immune cells, blood vessels, fibroblasts and extracellular matrix in the process of metastasis should also be considered, however, further investigations are required to clarify the mechanisms.

In conclusion, we demonstrated new findings that FF-10502 is significantly more efficacious than gemicitabine in a variety of clinically relevant mouse models of pancreatic cancer, including PDX models, which may be explained by inhibition of dormant cancer cells through a higher inhibition of polβ, unlike gemicitabine. A clinical trial of FF-10502 is currently underway in the United States to assess the usefulness of FF-10502 in patients with solid tumors, including pancreatic tumors. FF-10502 is expected to be a promising agent for pancreatic cancer with acquired resistance to gemicitabine.

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**Table 3**

Comet assay of DDI treatment in combination with FF-10502 or gemicitabine in human pancreatic SUIT-2 cells under serum starvation

Percentage of fluorescence intensity in tail DNA region per whole DNA is expressed as % tail DNA. The experiment was performed in duplicate, and the mean % tail DNA was calculated.

| Group                  | No DDIs | 5 μmol/l Cisplatin | 300 μmol/l H2O2 | 200 μmol/l TMZ |
|------------------------|---------|---------------------|-----------------|---------------|
|                        | 4 h     | 24 h                | 4 h             | 24 h          |
| Vehicle control (0.1% DMSO) | 5.5 | 7.4                  | 12.1 | 11.3       | 38.9 | 6.1 | 40.3 | 39.5 |
| FF-10502 100 nmol/l    | 8.9 | 17.1                 | 20.1 | 45.1       | 93.1 | 89.7 | 71.8 | 93.7 |
| FF-10502 1000 nmol/l   | 6.8 | 22.2                 | 19.9 | 49.7       | 93.8 | 95.8 | 88.1 | 97.4 |
| Gemicitabine 100 nmol/l| 5.6 | 6.5                  | 19.5 | 13.1       | 37.4 | 6.9  | 39.0 | 39.5 |
| Gemicitabine 1000 nmol/l| 5.2 | 6.8                  | 13.4 | 15.9       | 43.2 | 7.5  | 48.0 | 38.6 |

TMZ, temozolomide.
