MOLECULAR CANCER RESEARCH | CELL FATE DECISIONS

DNA Replication Stress Induced by Trifluridine Determines Tumor Cell Fate According to p53 Status

Yuki Kataoka1,2, Makoto Iimori1, Ryo Fujisawa3, Tomomi Morikawa-Ichinose4, Shinichiro Niimi5, Takeshi Wakasa1,2, Hiroshi Saeki6,7, Eiji Oki6, Daisuke Miura4,8, Toshiki Tsurimoto3, Yoshihiko Maehara5,6,9, and Hiroyuki Kitao1,5

ABSTRACT

DNA replication stress (DRS) is a predominant cause of genome instability, a driver of tumorigenesis and malignant progression. Nucleoside analogue-type chemotherapeutic drugs introduce DNA damage and exacerbate DRS in tumor cells. However, the mechanisms underlying the antitumor effect of these drugs are not fully understood. Here, we show that the fluorinated thymidine analogue trifluridine (FTD), an active component of the chemotherapeutic drug trifluridine/tipiracil, delayed DNA synthesis by human replicative DNA polymerases by acting both as an inefficient deoxynucleoside triphosphate source (FTD triphosphate) and as an obstacle base (trifluorothymidine) in the template DNA strand, which caused DRS. In cells, FTD decreased the thymidine triphosphate level in the dNTP pool and increased the FTD triphosphate level, resulting in the activation of DRS-induced cellular responses during S-phase. In addition, replication protein A-coated single-stranded DNA associated with FancD2 and accumulated after tumor cells completed S-phase. Finally, FTD activated the p53-p21 pathway and suppressed tumor cell growth by inducing cellular senescence via mitosis skipping. In contrast, tumor cells that lost wild-type p53 underwent apoptotic cell death via aberrant late mitosis with severely impaired separation of sister chromatids. These results demonstrate that DRS induced by a nucleoside analogue-type chemotherapeutic drug suppresses tumor growth irrespective of p53 status by directing tumor cell fate toward cellular senescence or apoptotic cell death according to p53 status.

Implications: Chemotherapeutic drugs that increase DRS during S-phase but allow tumor cells to complete S-phase may have significant antitumor activity even when functional p53 is lost.

Introduction

Accurate DNA replication is fundamental to faithful genome duplication and cellular proliferation (1). Obstacles that perturb DNA replication process induce cellular stress termed DNA replication stress (DRS). Tumor cells often show dysregulation of DNA replication and sustained proliferation signaling, which leads to DNA damage and chronic DRS (2). Conventional chemotherapy exerts its cytotoxic effects by causing DNA damage and simultaneously promotes DRS by perturbing the DNA replication process (3). DRS induced by chemotherapy triggers the activation of cellular response pathways for survival in tumor cells; however, loss or suppression of the stress response can increase the susceptibility of tumor cells to catastrophic failure of proliferation. Thus, exploiting DRS is a feasible approach for cancer therapy (4).

DRS triggers various cellular responses in tumor cells. DRS activates ataxia telangiectasia and Rad3-related (ATR) kinase, which is recruited to replication protein A (RPA)-coated single-stranded DNA (ssDNA) at the stalled replication fork and transduces signals to numerous downstream targets (e.g., Chk1 is the main effector kinase) via phosphorylation to activate the S-phase checkpoint (5). Thus, ATR and Chk1 preserve genome integrity by stabilizing the stalled fork and preventing origin firing. DRS also activates the Fancd2 monoubiquitination, which plays a crucial role in stabilizing stalled replication forks (6). In cases of moderate DRS, however, tumor cells can proceed to G2 and M phases with under-replicated DNA (UR-DNA) at hard-to-replicate DNA breaks. Chk1 controls DNA re-replication, preventing post-replication gaps (7). DRS activates bcl-2 family members and promotes cellular senescence (8). DNA replication stress activates p53 and induces cellular senescence-like growth arrest (9). Cellular senescence in tumor cells is also induced by anticancer chemotherapy and improves long-term outcomes (10).

Nucleoside analogue-type chemotherapeutic drugs, such as gemcitabine and cytarabine, are structurally similar antimetabolites with a broad range of action, and they are clinically active in both solid tumors and hematologic malignancies (11). These drugs are efficiently transported into the cytoplasm of tumor cells, rapidly phosphorylated to

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Y. Kataoka and M. Iimori contributed equally to this article.

Current address for R. Fujisawa: MRC PPU, Sir James Black Centre, School of Life Sciences, University of Dundee, Dundee, United Kingdom.

Corresponding Author: Hiroyuki Kitao, Kyushu University, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan. Email: hkitao@phar.kyushu-u.ac.jp

Mol Cancer Res 2020;18:1354–66
doi: 10.1158/1541-7786.MCR-19-1051
© 2020 American Association for Cancer Research.
triprophosphate forms, incorporated into DNA during normal DNA synthesis by replicative DNA polymerases, and compromise DNA replication. Trifuridine (FTD) is a fluorinated thymidine analogue included in the clinically approved chemotherapy drug called trifuridine/tipiracil (FTD/TP); also named TAS-102; refs. 14, 15). FTD is massively incorporated into DNA without detectable DNA strand breaks, induces Chk1 phosphorylation at serine 345 (pS345 Chk1), a specific site phosphorylated by ATR kinase upon DRS (5), and activates the p53-p21 pathway, leading to sustained cell-cycle arrest at a phase with 4N DNA content (16). Although it has already been reported that FTD induces cell death independently of p53 (17), the mechanism underlying the induction of DRS by FTD and its contribution to subsequent cell fate decisions remains to be fully elucidated.

In this study, we demonstrate that FTD, a nucleoside analogue, delayed but did not terminate DNA replication in vitro. At the cellular level, FTD stalled replication forks and generated DNA lesions including ssDNA, which persisted after S-phase was completed. In tumor cells, FTD led to the activation of the p53-p21 pathway, mitosis skipping, and persistent growth arrest characterized as cellular senescence. On the other hand, in p53 knock-out tumor cells, FTD led to aberrant mitosis with severely impaired sister chromatid separation, which caused apoptotic cell death. These data indicate that DRS induced by FTD treatment generates an antitumor effect by changing tumor cell fate according to p53 status.

Materials and Methods

Cell culture and reagents

HCT-116 cells were purchased from ATCC in 2011. DLD1 cells were provided by Taiho Pharmaceutical Co. Ltd. (18). A549 cells were provided by Dr. M. Takeda (Kyushu University, Fukuoka, Japan; ref. 16). All cells were authenticated by short tandem repeat analysis (Biologica Co.) in 2018 and confirmed negative for Mycoplasma infection with the MycoAlert Mycoplasma Detection Kit (Lonza) in 2020. HCT-116 and A549 cells were cultured in DMEM, and DLD1 cells were cultured in RPMI1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO2. Cells were stored in liquid nitrogen within two cell passages and the assays were performed on cells within 2 months after thawing. siRNA transfection was performed using RNAiMax reagent (Thermo Fisher Scientific). The siRNA sequences used were: FomD2 (5'-GGAGAUGUAGAGUCUAGCUA-3'), and Luc (GL3: 5'-CUUAGCCUGUAGUA-CUUCGA-3').

DNA fiber analysis

DNA fiber analysis was performed as described previously (19) with some modifications. For nascent DNA labeling, DLD1 cells were cultured in the presence of 20 µmol/L CdU for 20 minutes, washed twice with fresh medium, and cultured in the presence of 20 µmol/L IdU or 20 µmol/L FTD for the indicated times. After double labeling, cell suspensions were spotted on slides, air-dried, and lysed with cell lysis solution (200 µmol/L Tris-HCl, pH7.5, 50 µmol/L EDTA, and 0.5% SDS). Following cell lysis, the slides were tilted to 15° to allow the DNA fibers to spread along the slides. After fixation with methanol/acetate acid (3:1) and DNA denaturation with 2.5 N HCl, the DNA fibers on slides were immunostained with two different anti-BrdU antibodies (Supplementary Table S1), anti-mouse IgG conjugated with Alexa Fluor 568, and anti-rat IgG conjugated with Alexa Fluor 488 (Thermo Fisher Scientific) at 1:400 dilution. Slides were mounted with Vectashield (H-1000; Vector Laboratories).

In vitro DNA polymerase assay

The proteins used for the in vitro DNA polymerase assay (Supplementary Fig. S1C) were purified as described previously (20–22). Human DNA polymerase δ/ε activity was measured with respect to the incorporation of [α-32P]dAMP. The reaction mixture (10 µL) contained 25 mmol/L Hepes-NaOH (pH 7.8), 0.1 mg/mL BSA, 0.5 mmol/L DTT, 10 mmol/L Mg(CH3COOH)2, 2 mmol/L ATP, 100 µmol/L each of dGTP, dCTP, dATP, the indicated concentrations of dTTP or dTTP analogue, 0.625–1.25 µmol/L (α-32P) dATP, 12.4 fmol (90 pmol for nucleotides) of singly primed M13mp18 DNA (the 90-mer primer: 5′-AGGCCGTCGATTAACACCGCTGACACGTGACGTCAGAGGACCGCCTGCAAC-ATGGTGAGCCAGCAGCAAATGAAAATCTAAA-GCCTACCTTGCTGACACTCA-3′ is complementary to nucleotide positions 4833 and 4922). 3.5 pmol replication protein A for Pol α or 3.5 pmol ssDNA binding protein for Pol ε, 1 pmol proliferating cell nuclear antigen (PCNA), 60 fmol replication factor C, and the indicated amounts of polymerase. After incubation at 37°C for 30 minutes, reaction mixtures were immediately chilled on ice, and 7 µL samples were spotted on Whatman DE81 paper (GE Healthcare). The unincorporated nucleotides were washed four times with 0.5 mol/L Na2HPO4, and the incorporated (α-32P) dAMP adsorbed onto the paper was measured by Cherenkov counting with a liquid scintillation counter (Beckman Coulter).

To measure DNA synthesis on the oligonucleotides, the 5′ end of the oligonucleotide primer was radioactively labeled with [γ-32P] ATP, annealed with the template oligonucleotide, and subjected to in vitro DNA polymerase reaction at 37°C for the indicated times. The reaction was stopped by adding a bromophenol blue/xylene cyanate-formamide EDTA solution. After boiling at 95°C for 3 minutes, the samples were loaded onto 15% acrylamide gels containing 7% urea and electrophoresed at 30W for 60 minutes. The gels were fixed with 15% methanol/15% acetic acid solution for 10 minutes, rinsed with tap water, and dried on 3MM paper. Radioactivity was detected using BAS2000 (GE Healthcare).

Quantification of dTTP, FTD-TP, and BrdUTP using LC/triple-stage quadrupole mass spectrometry

Intracellular dTTP, FTD-TP, and BrdUTP were quantified using LC-QqQ-MS (LCMS-8040; Shimazu) as described previously (23). FTD-TP and BrdUTP were detected with optimized selective reaction-monitoring transitions in negative ionization mode as follows: FTD-TP: precursor ion [m/z]/product ion [m/z] = 535/159, 535/79, and 535/257 and BrdUTP: precursor ion [m/z]/product ion [m/z] = 546.5/159.

Generation of TP53-deficient HCT-116 cells

First, a 20-mer sgRNA target sequence (5′-CTCAGAGGGGGGTCATGAGGC-3′) at exon 2 of the TP53 gene was designed (24) and cloned into pX330 (Addgene #42230), which was a gift from Dr. Feng Zhang (25). The donor DNA plasmid was constructed from the PCR fragment (~1,300-bp of the TP53 genomic region including the sgRNA target sequence amplified by PCR (forward: 5′-ACTA- TATCCTTGTAAACAGGGGTTGGAGGACG-3′; reverse: 5′-AAGGG- TGAAGGAGGATCCCAATGTCCCAAC-3′) in pCR4-TOPO (Thermo), and its 130-bp Bam HI fragment that includes the sgRNA target sequence was replaced with the 2,500-bp Bam HI fragment containing a puromycin-resistant gene cassette (26). The above two plasmids were cotransfected into HCT-116 cells using 4D
Figure 1.
FTD retards replication fork progression. A, DNA fiber analysis; DLD1 cells were cultured in the presence of 20 μmol/L CldU for 20 minutes and either of 20 μmol/L IdU (top) or 20 μmol/L FTD (bottom) for 20 minutes. Representative images are enlarged. Scale bars, 10 μm (left) and 5 μm (enlarged). B, Scatter plot of relative IdU- or FTD-tract lengths normalized by the CldU-tract lengths for individual replication forks in A. Red lines denote median and blue whiskers extend to the quartiles. Mann–Whitney U test. *, P < 0.05; ***, P < 0.001; ****, P < 0.0001. C, Western blot analysis; DLD1 cells were cultured in the presence of 20 μmol/L nucleoside analogues for 1 hour. D, The in vitro DNA synthesis rate of replicative polymerases Polδ (left) and Polε (right), in the presence of dTTP, BrdUTP, or FTD-TP. (Continued on the following page.)
Nucleofector (Lonza). The puromycin-resistant clones were screened by genomic PCR and sequencing.

**Generation of fluorescent ubiquitination–based cell-cycle indicator-expressing cells by lentiviral infection**

cDNA encoding mKO2-hCdt1 (aa 30–120) or mAG1-hGeminin (aa 1–110) amplified from the pFucci-G1 Red plasmid (AM-V9003) or pFucci-S/G2-M Green plasmid (AM-V9016; MBL), respectively, was cloned into the pENTR D-TOPo vector (Thermo Fisher Scientific). Each plasmid was mixed with pLent6.4/R4R2/V5-DEST and pENTR 5’/EF1aTer and recombined using LR Clonase II Plus enzyme (Thermo Fisher Scientific). The lentiviruses were produced using the ViraPower Lentiviral Expression System (Thermo Fisher Scientific). To establish HCT-116-fluorescent ubiquitination–based cell-cycle indicator (Fucci) or HCT-116 p53+/−Fucci cells, cells were infected with both lentiviruses encoding mKO2-hCdt1 (30–120) and mAG1-hGeminin (1–110) at a multiplicity of infection of 1 each. The infected cells were first selected by blastcidin (Thermo Fisher Scientific) treatment. The surviving cells were sorted in two steps using BD FACS Aria SORP (BD Biosciences) as follows: cells emitting red fluorescence were sorted and cultured for several days, and then cells emitting green fluorescence were sorted.

**Western blot analysis**

Western blot analysis was performed as described previously (16). The antibodies used are listed in Table S1. Chemiluminescence was detected using LAS 4000 mini (GE Healthcare).

**Immunofluorescence**

To visualize mitotic cells, cells were rinsed in PBS at 37°C, fixed in 4% paraformaldehyde (PFA) for 15 minutes at 37°C (27), permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes at 37°C, and blocked in PBS containing 2% BSA for 30 minutes at room temperature. To visualize nuclear protein, cells were fixed with 3% PFA, 2% sucrose, and 0.5% Triton X-100 for 30 minutes on ice (28), permeabilized in PBS containing 0.1% NP-40, and blocked with PBS containing 3% BSA for 20 minutes at room temperature. To visualize FTD incorporated into DNA, cells were fixed with 70% ethanol, depurinated with 1.5 N HCl, and blocked with PBS containing 5% goat serum, 0.3% Triton X-100 for 1 hour at room temperature (29). The cells were then incubated overnight at 4°C with the antibodies listed in Supplementary Table S1. Secondary antibodies conjugated to Alexa Fluor 488, 568, or 647 (Thermo Fisher Scientific) were used. After washing in PBS containing DAPI for 5 minutes, cells were mounted on coverslips using ProLong Diamond or ProLong Glass (Thermo Fisher Scientific).

**In situ proximal ligation assay**

Cells were fixed in 3% PFA, 2% sucrose, and 0.5% Triton X-100 for 30 minutes on ice, permeabilized with PBS containing 0.25% Triton X-100 for 30 minutes at room temperature, blocked with PBS containing 3% BSA, and incubated overnight at 4°C with anti-FanCD2 (NB100-182; Novus Biologicals) and anti-RPA32 (ab2175, Abcam) antibodies. PLA was performed with reagents from Duolink PLA technology in accordance with the manufacturer’s instructions (Sigma).

**Image acquisition**

For fixed-cell experiments, fluorescence image acquisitions were performed using a Nikon A1R confocal imaging system or N-SIM super resolution imaging system controlled by NIS Elements software (Nikon). The objective lens was an oil immersion CFI SR ApoTIRF 100× NA 1.49 lens, an oil immersion Plan-Apo 100× NA 1.45 lens, or a Plan-Apo 40× NA 0.95 lens (Nikon). Images were acquired as Z-stacks at 0.2-μm or 0.12-μm intervals with a confocal or a super-resolution microscope, respectively, and maximum-intensity projections were generated using NIS Elements software (Nikon). The fluorescence intensity of RPA32 nuclear foci was quantified using Image J (NIH, Bethesda, MD) for each stack in the images with maximum-intensity projections. For live-cell imaging of cell-cycle progression, HCT-116-Fucci or HCT-116 p53+/−Fucci cells were imaged in a chambered coverglass (Matsunami) containing phenol red-free DMEM (Gibco). Live-cell imaging was performed as described previously (27). The duration of the cell-cycle phases was calculated manually. For the quantification of nuclear foci and PLA signals, fluorescence images were acquired using a BZ-X800 (Keyence) with Plan-Apo 40× NA, and nuclear foci were counted using Hybrid Cell Count Software (Keyence). For the quantification of FTD incorporation into DNA, fluorescence images were acquired using a Cytell (GE Healthcare) and analyzed using In Cell Investigator software (GE Healthcare).

**Animals and evaluation of antitumor activity in vivo**

All animal studies were performed according to the guidelines and with the approval of the institutional Animal Care and Use Committee of Taiho Pharmaceutical Co., Ltd. Ethical approval (March 5, 2019) was obtained prior to conducting the animal experiments. Male nude mice (CLEA Japan) were housed under specific pathogen-free conditions, with food and water provided *ad libitum*. The animals were quarantined for 1 week and then subcutaneously implanted with 1 × 10^7 HCT-116 or HCT-116 p53+/−cells on day 0. The mice were separated into each treatment group on day 3 so that the average body weight of each group was equivalent. FTD/TPI was prepared by mixing FTD and TPI at a molar ratio of 1:0.5 in 0.5% HPMC solution. FTD/TPI (FTD: 200 mg/kg/day) was administered orally twice daily from days 3–7, days 10–14, and days 17–21 at approximately 6-hour intervals. For the control group, vehicle (0.5% HPMC solution) was administered at 10 ml/kg.

**Statistical analysis**

The statistical analysis was performed using GraphPad Prism (GraphPad Software) or EXSUS (CAC Croit Corp.) software. The
FTD impedes replicative DNA polymerases in vitro

We hypothesized that FTD triphosphate (FTD-TP) would be incorporated into DNA during replication catalyzed by replicative DNA polymerases (Polβ and Polε) with low efficiency, which could slow the elongation of the nascent DNA strand. To test this possibility, DNA synthesis was evaluated by measuring the incorporation of radioactivity ([32P]dATP) in an in vitro reconstituted DNA replication assay using purified proteins (Supplementary Fig. S1C–S1E). The [32P]dATP incorporation rate was significantly lower in the presence of FTD–TP than in the presence of dUTP or BrdUTP (Fig. 1D). In addition, the low [32P]dATP incorporation rate in the presence of FTD-TP was almost completely rescued when 50% of FTD-TP was replaced by dTTP (Fig. 1E). These results indicate that the incorporation rate of FTD–TP into the nascent DNA strand was less efficient than that of dTTP or BrdUTP.

To identify the specific sequence in the template DNA strand at which the synthesis of the nascent DNA strand is impeded in the presence of FTD–TP, we performed an in vitro DNA replication assay with a defined DNA template. Synthesis of the nascent DNA strand in the presence of FTD–TP was strongly and specifically impeded at positions of adenine-rich sequences (Fig. 1F).

We further examined whether the elongation of the nascent DNA strand was retarded when Polβ or Polε encounter FTD in the template DNA strand. Elongation was significantly suppressed when either Polβ or Polε was used to replicate a template DNA strand containing five repetitive FTDs at the 3′ vicinity of the primer end (Fig. 1G). Collectively, these results indicate that FTD induced DRS by impeding the progression of replicative DNA polymerases during its incorporation into the nascent strand DNA as well as when FTD was present in the template DNA strand.

FTD rapidly decreases dTTP and increases FTD-TP in the cellular dNTP pool

Next, we examined whether FTD–TP was produced when cells were cultured in the presence of FTD. In HCT-116 cells cultured in the presence of various concentrations of FTD for 60 minutes, FTD incorporation was detected (Supplementary Fig. S2A), and dTTP decreased and FTD triphosphate (FTD–TP) increased in a concentration-dependent manner (Supplementary Fig. S2B and S2C). In HCT-116 cells cultured in the presence of FTD, dTTP decreased rapidly as FTD–TP increased in the cellular dNTP pool (Fig. 2A and B). At this time point, FTD was being continuously incorporated into DNA (Fig. 2C) and induced pS345 Chk1 (Fig. 2D). When FTD was removed from the medium, dTTP recovered rapidly as FTD–TP disappeared from the dNTP pool (Fig. 2E and F). Furthermore, thymidine suppressed FTD incorporation into DNA in a concentration-dependent manner (Supplementary Fig. S2D), and thymidine as well as BrdU decreased FTD–TP and suppressed FTD-induced pS345 Chk1 (Supplementary Fig. S2E–S2H). These data indicate that FTD–TP was produced when cells were cultured in the presence of FTD and that it activated the DNA damage response during its incorporation into DNA.

FTD induces FancD2 monoubiquitination during S-phase and results in the accumulation of persistent ssDNA

Next, we monitored cellular responses in synchronized HCT-116 cells cultured in the presence of FTD (Supplementary Fig. S3A and S3B). FTD induced pS345 Chk1 and FancD2 monoubiquitination as cells proceeded through S-phase (Fig. 3A), indicating the activation of ATR kinase and FA pathway in response to FTD. In contrast, FancD2 monoubiquitination was induced only modestly during S-phase in the absence of FTD (Supplementary Fig. S3C–S3E). After completing S-phase, cells showed accumulation of p53 and p21 as they proceeded into the next cell-cycle phase with a 4N DNA content, which was accompanied by a decrease in the Cyclin B1 level (Fig. 3A). Because nuclear retention and proteasome-mediated degradation of Cyclin B1 occurs in a p53- and p21-dependent manner at G2 phase when cells are permanently withdrawn from the cell cycle (30), these data indicate that FTD activated p53-p21 pathway at G2 phase as a consequence of DRS and enforced permanent exit from the cell cycle.

The accumulation of ssDNA is considered a hallmark of DRS and causes genome instability (31). To investigate whether FTD treatment results in the accumulation of ssDNA, we communostained the ssDNA binding protein RPA32 and FancD2 in FTD-treated HCT-116 cells. We found a modest increase in the number of RPA32 nuclear foci at 24 hours (Fig. 3B and D), when most cells were in S-phase (Supplementary Fig. S3F). This was accompanied by pS345 Chk1 and FancD2 monoubiquitination (Supplementary Fig. S3G) and the formation of pan-nuclear FancD2 foci (Fig. 3B and C). Intriguingly, the number of intense RPA32 nuclear foci were increased at 48 hours (Fig. 3B and D), when most cells had a 4N DNA content (Supplementary Fig. S3F), and p53 and p21 were induced (Supplementary Fig. S3G). These data suggest that the FTD-treated cells accumulated ssDNA, which was associated with replication fork stalling during S phase, and the ssDNA increased after cells completed the S-phase.

In human primary cells, FancD2 is required to restrain DNA synthesis under DRS and to prevent the accumulation of ssDNA and the induction of p21 (32). FancD2 and RPA32 were colocalized at FTD-induced nuclear foci (Fig. 3B). Furthermore, FTD increased significantly the number of PLA signals of FancD2 and RPA32 (Fig. 3E; Supplementary Fig. S3H and S3I), confirming that FTD increased their association, bringing them into close proximity in the nucleus. We hypothesized that FancD2 would function near RPA-coated ssDNA after FTD treatment. To investigate this, we knocked
Figure 2. Effect of FTD on the cellular and metabolomic states.
Relative amounts of cellular dTTP (A) and FTD-TP (B). The relative scores were calculated by considering the average amount at 0 minutes (A, 70.6 ± 313 pmol/10^6 HCT-116 cells, n = 3) or 60 minutes (B, 57.7 ± 471 pmol/10^6 HCT-116 cells, n = 3) of 5 μmol/L FTD treatment as 1, respectively. ND: not detectable.

Consider the average amount at 240 minutes of FTD incorporation into DNA. The relative scores were calculated by considering the average amount at 240 minutes of 5 μmol/L FTD treatment as 1. D, Western blot analysis; HCT-116 cells were cultured in the presence of 5 μmol/L FTD and harvested at the indicated time points. pS345 Chk1, Chk1 phosphorylation at Ser345. Relative amounts of cellular dTTP (E) and FTD-TP (F) after the change to drug-free media. The relative scores were calculated by considering the average amount at 0 minutes (E) or 5 μmol/L FTD 30 minutes (F) as 1, respectively. Error bars, SD of three independent experiments.

FTD induces senescence in tumor cells with wild-type p53 and apoptosis in p53 knockout tumor cells

We previously showed that FTD induces p53-dependent sustained cell-cycle arrest at the cell-cycle phase with a 4N DNA content (16). To elucidate the role of p53 in the FTD-induced DNA damage response, we generated isogenic TP53 gene knockout HCT-116 cell lines with similar FTD sensitivity using the CRISPR/Cas9 system (Supplementary Fig. S3J and S3K). In the presence of FTD, cell proliferation was similarly suppressed in HCT-116 and HCT-116 p53⁻/⁻ cells (Fig. 4B). HCT-116 cells showed accumulation of p53 and p21 and almost undetectable expression of Cyclin B1 and Cyclin A proteins on day 3 at the time when accumulation of cyclin D1 was observed (Fig. 4B), suggesting that a significant proportion of cells were in G1 phase. In HCT-116 p53⁻/⁻ cells, however, p21 and cyclin D1 did not accumulate, and Cyclin B1 and Cyclin A levels did not decrease (Fig. 4B). Furthermore, similar results were obtained using A549 (p53 wild-type) and DLD1 (p53 mutant) cells (Supplementary Fig. S4C–S4E). These data indicate that FTD activated the p53-p21 pathway and suppressed the growth of cells expressing wild-type p53, resulting in the accumulation of cells in G1 phase, whereas in cells without p53, FTD caused tumor cell death.

Chronic DRS activates p53 and induces cellular senescence-like growth arrest (11). Consistently, on day 3 of FTD treatment, most HCT-116 cells were senescence-associated β-galactosidase (SA-β-gal)-positive, whereas only a limited population of HCT-116 p53⁻/⁻ cells showed this phenotype (Fig. 4C). Similar results were obtained with A549 and DLD1 cells (Supplementary Fig. S4F). By contrast, on day 6 of FTD treatment, the number of cells in the sub-G1

Published OnlineFirst May 28, 2020; DOI: 10.1158/1541-7786.MCR-19-1051

Mol Cancer Res; 18(9) September 2020 1359

AACRJournals.org
were cultured in the presence of 3 μmol/L FTD for indicated time. Representative colocalizing foci were measured in three independent experiments. The horizontal lines indicate medians, and the boxes and whiskers indicate the interquartile and minimum/maximum ranges, respectively. Kruskal-Wallis test. * * * *; P < 0.0001; ns, not significant. F. Immunofluorescence images of FanCD2 and RPA32 in siFanCD2- and FTD-treated HCT-116 cells, which were cultured in the presence of 3 μmol/L FTD 48 hours; scale bar, 10 μm. G. Box plots of the intensity of each RPA32 focus in F. The integrated intensity of each focus was plotted. Two-hundred foci were measured in three independent experiments. The horizontal lines indicate medians, and the boxes and whiskers indicate the interquartile and minimum/maximum ranges, respectively. Mann-Whitney U-test (*** *; P < 0.0001).

Figure 3.
DNA damage response to FTD in HCT-116 cells. A, Western blot; DNA damage response of synchronized HCT-116 cells. A, Asynchronous; R, RO-3306 arrested. Cell-cycle phases at each time point are indicated above. B, Immunofluorescence images of FanCD2 and RPA32 in HCT-116 cells, which were cultured in the presence of 3 μmol/L FTD for indicated time. Representative colocalizing foci are indicated by arrows; scale bar, 10 μm. Box plots of the number of FanCD2 foci (C), and RPA32 foci (D) in B. E, Box plots of the number of proximal ligation assay (PLA) signals between FanCD2 and RPA32 per each nucleus of HCT-116 cells, which were cultured in the presence of 3 μmol/L FTD for indicated time. Two hundred nuclei for each time point were counted in three independent experiments. The horizontal lines indicate medians, and the boxes and whiskers indicate the interquartile and minimum/maximum ranges, respectively. Kruskal-Wallis test. * * * *; P < 0.0001; ns, not significant. F, Immunofluorescence images of FanCD2 and RPA32 in siFanCD2- and FTD-treated HCT-116 cells, which were cultured in the presence of 3 μmol/L FTD 48 hours; scale bar, 10 μm. G, Box plots of the intensity of each RPA32 focus in F. The integrated intensity of each focus was plotted. Two-hundred foci were measured in three independent experiments. The horizontal lines indicate medians, and the boxes and whiskers indicate the interquartile and minimum/maximum ranges, respectively. Mann-Whitney U-test (*** *; P < 0.0001).
Figure 4.
Outcomes of HCT-116 and HCT-116 p53−/− cells cultured in the presence of FTD. A, Growth curve; cell growth was measured by crystal violet staining on the indicated days. Error bars represent SD of three independent experiments. B, Western blot analysis of RIPA extracts; HCT-116 and HCT-116 p53−/− cells were cultured in the presence of 3 μmol/L FTD either continuously (continuous) or for 3 days (wash at day 3) for the indicated days and harvested. P represents a positive control (HCT-116 cells, which were cultured in the presence of 3 μmol/L FTD for 3 days). C, SA-β-gal activity of HCT-116 and HCT-116 p53−/− cells on day 3; The black bar represents 50 μm. The graph shows the percentage of SA-β-gal-positive cells. Error bars represent the SD of three independent experiments. D, Sub-G1 population. HCT-116 and HCT-116 p53−/− cells were cultured in the presence of 3 μmol/L FTD and harvested on the indicated days. Ethanol-fixed samples were stained with propidium iodide and the percentage of sub-G1 cells was measured. Error bars, the SD of three independent experiments. E, Western blot analysis of whole-cell extracts; HCT-116 and HCT-116 p53−/− cells were cultured in the presence of 3 μmol/L FTD as shown in B. Growth curve of HCT-116 (F) and HCT-116 p53−/− (G) xenografts. Error bars represent the SD of 6 individual mice. Statistical analysis was done at day 24. Unpaired t test (**, P < 0.01; ***, P < 0.001).
Figure 5.
Single-cell live imaging of Fucci cells in response to FTD. A, Representative images of HCT-116-Fucci and HCT-116 p53−/−-Fucci cells, which were cultured in the presence or absence of 3 μmol/L FTD. Arrowheads indicate identical cells at each time point. B, Cell-cycle phase and duration in HCT-116-Fucci and HCT-116 p53−/−-Fucci cells, which were cultured in the presence or absence of 3 μmol/L FTD. Each bar represents one cell that was in G1 phase (red color) at the start point. C, analysis terminated. Scattered plots of duration of the first S-G2 phase and the second S-G2 phase (D), the first M phase and the second M phase (F). Red lines and blue whiskers denote means and SD, respectively. Mann–Whitney U-test (†, P < 0.05; ††, P < 0.01; †††, P < 0.0001; ns, not significant; ND, not detected).

Discussion

In this study, we elucidated the mechanism underlying the antitumor effect of the fluorinated thymidine analogue-type chemotherapeutic drug, FTD. FTD treatment resulted in the replacement of dTTP in the dNTP pool with FTD-TP, which slowed DNA synthesis by replicative DNA polymerases. Thus, FTD stalled replication forks, activated ATR-dependent DNA damage responses, and resulted in the accumulation of RPA-coated ssDNA, which persisted even after cells completed the S-phase. FancD2 suppressed the FTD-induced ssDNA accumulation. Subsequently, FTD activated p53 and p21, thereby inducing cellular senescence. In the absence of p53, FTD triggered apoptotic cell death by inducing aberrant mitosis associated with severely unseparated sister chromatids. Because tumor cells show high levels of DRS and depend on their response to DRS, exploiting DRS is a feasible approach for cancer therapy (4). As a chemotherapeutic drug, FTD exploits cellular DRS and exerts antitumor effects irrespective of the p53 status of tumors (Fig. 7).

In addition, tipiracil hydrochloride, as a component of FTD/TPI, inhibits thymidine phosphorylase and exerts an antiangiogenesis effect (36), which may further enhance the cytotoxic effect of FTD.

Biochemical analysis of the in vitro reconstituted DNA replication process using human Polδ and Polε revealed the unique properties of FTD as a nucleoside analogue. First, as a component of the dNTP pool, FTD-TP can replace dTTP during DNA synthesis at the replication fork; however, FTD-TP incorporation was inefficient (Fig. 1D and F). Another intriguing property of FTD-TP is that FTD incorporation did not terminate DNA polymerization (Fig. 1D), allowing its continuous incorporation into DNA to produce FTD-containing DNA strands. In contrast, other widely-used nucleoside analogue-type chemotherapeutic drugs, such as gemcitabine and cytarabine, terminate DNA polymerization in the vicinity of their incorporation in a cell-free system (13), strongly inhibit DNA synthesis at the cellular level (37), and are incorporated into DNA to a lower extent than FTD (38). Second, as a component of the template DNA strand, FTD constitutes a continuous obstacle to DNA polymerization (Fig. 1G). This property may explain the severe extension of the second S-G2 phase after FTD addition (Fig. 5), because DNA synthesis at the second S-phase would have to proceed with the FTD-containing template DNA strand in the
A, Representative immunofluorescence images of anaphase HCT-116 p53<sup>−/−</sup> cells, which were either untreated (Normal, and UFB) or cultured in the presence of 3 μmol/L FTD and 9 μmol/L RO-3306 for 16 hours and released into fresh media for 50 minutes (Chr. Bridge, and Chr. Bridge+UFB). B, Quantitative data of A. Error bars represent SD of three independent experiments. Unpaired t test. ***P < 0.001, **P < 0.01. C, Representative super-resolution immunofluorescence images of anaphase HCT-116 p53<sup>−/−</sup> cells, which were cultured in the presence of 3 μmol/L FTD for 60 hours. The centromeres (ACA) captured by spindles are indicated by yellow arrowheads. Sister chromatid pairs with entangled chromosome arms are indicated by white arrowheads. Enlarged images of rectangles with dashed lines are shown in the inlets. D, Quantitative data of abnormal anaphase. Error bars, SD of three independent experiments. Unpaired t test (***, P < 0.001).

Figure 6. Aberrant chromosomal structures of HCT-116 p53<sup>−/−</sup> cells in response to FTD. A, Representative immunofluorescence images of anaphase HCT-116 p53<sup>−/−</sup> cells, which were either untreated (Normal, and UFB) or cultured in the presence of 3 μmol/L FTD and 9 μmol/L RO-3306 for 16 hours and released into fresh media for 50 minutes (Chr. Bridge, and Chr. Bridge+UFB). B, Quantitative data of A. Error bars represent SD of three independent experiments. Unpaired t test. ***P < 0.001, **P < 0.01. C, Representative super-resolution immunofluorescence images of anaphase HCT-116 p53<sup>−/−</sup> cells, which were cultured in the presence of 3 μmol/L FTD for 60 hours. The centromeres (ACA) captured by spindles are indicated by yellow arrowheads. Sister chromatid pairs with entangled chromosome arms are indicated by white arrowheads. Enlarged images of rectangles with dashed lines are shown in the inlets. D, Quantitative data of abnormal anaphase. Error bars, SD of three independent experiments. Unpaired t test (***, P < 0.001).

presence of FTD-TP in the dNTP pool. Third, FTD incorporated into the DNA of tumor cells is retained for a prolonged period (39). This property would cause persistent disturbance of the replication process and a long-term tumor cytotoxicity, which may underlie the sustained growth-suppressive effect and prolonged survival observed in a xenograft mouse model exposed to limited courses of FTD/TPI (38). The dual perturbation of DNA replication caused by the inefficient incorporation of FTD during DNA synthesis and the persistent DNA polymerization roadblock caused by the presence of FTD on the template DNA strand are probably key properties of FTD.

Because FTD is a thymidine analogue, inefficient DNA replication should occur preferentially at AT-rich genomic loci (40). Recently, genome-wide analysis revealed that large homopolymeric dA/dT tracts are preferential sites of polar replication fork stalling and collapse within early-replicating fragile sites, CFSs, and replication fork barriers at rDNA (41). FTD may exacerbate fork stalling at these fragile sites, retard S-phase progression, and increase ssDNA accumulation even after cells have completed the S-phase. Our previous study, however, did not detect an increase in DNA strand breaks in FTD-treated HCT-116 cells (16). FTD-induced ssDNA could be protected by the cooperative actions of proteins that accumulate in nuclear foci, such as FancD2 and the RPA complex (Fig. 3B). The RPA complex binds and stabilizes ssDNA (42) and FancD2 prevents excess ssDNA accumulation (Fig. 3F and G) (32). To repair or tolerate these abnormal DNA structures, homologous recombinational repair (BRCA1 or BRCA2), postreplication repair (Rad18) and translesional DNA synthesis (Rev3) may be involved, because vertebrate cells deficient in these factors show higher FTD sensitivity (43). Ultimately, the p53-p21 pathway is activated to avoid catastrophic collapse of chromosomes (11). On the other hand, in p53 knockout cells, FTD-induced ssDNA was probably converted to detrimental DNA strand breaks, as evidenced by the detection of γH2AX (Fig. 4E), via aberrant mitosis progression and the failure of sister chromatid separation, probably caused by interlinks between ssDNA located at chromosomal arms (Fig. 6C and D).

Cell analysis revealed that FTD suppressed the growth of tumor cells irrespective of p53 status. This cellular outcome differs from that induced by gemicitabine, cytarabine, and 5-fluorouridine, which induce p53-dependent apoptotic cell death, and loss of p53 confers drug resistance (44). The unique feature of FTD action is that it results in the p53-dependent divergence in cell fate at the G2–M phase transition, resulting in redirection of cell fate toward either cellular senescence or apoptotic cell death. The p53-mediated senescence, however, impairs the apoptotic response to chemotheraphy, and the senescent tumor cells show persistent mitogenic potential, which causes relapse (45). Recent studies indicate that establishment of senescence may reprogram tumor cells into a latent stem-like state, resulting in tumor cells that escape senescence having a more aggressive phenotype (46). Because FTD is a component of the chemotherapeutic drug FTD/TPI, it is critical to determine whether FTD-induced senescent tumor cells also acquire stemness and a persistent mitogenic potential. If that is the case, a strategy for evading FTD-induced senescence or for directing tumor cell fate toward apoptotic death should be given serious consideration.

FTD/TPI shows efficacy in the treatment of patients with gastrointestinal cancer who are refractory or intolerant of 5-FU-based therapy (14, 15). At the cellular level, acquired resistance to 5-FU does not confer FTD resistance and vice versa (18, 36, 47–49), possibly reflecting the distinct mechanism of action of each drug. The unique property of FTD demonstrated in this study may also confer distinct
cytotoxicity toward tumors that have acquired resistance to other conventional chemotherapeutic drugs. In addition, missense mutations, including both loss- and gain-of-function mutations, are frequently found in the TP53 gene locus of tumors obtained from patients (50). Whether the expression of mutant p53 affects the cellular response and tumor cell fate decision induced by FTD is an important issue that warrants further investigation.

Disclosure of Potential Conflicts of Interest
Y. Kataoka reports receiving other commercial support from Taiho Pharmaceutical Co., Ltd. during the conduct of the study. M. Iimori is a member of the Joint Research Department funded by Taiho Pharmaceutical Co. Ltd. in Kyushu University. T. Wakasa is an employee at Taiho Pharmaceutical Co. Ltd. E. Oki reports receiving personal fees from Taiho Pharmaceutical Co., Ltd. outside the submitted work. H. Kitao reports receiving grants from Taiho Pharmaceutical Co., Ltd. and The Ministry of Education, Culture, Sports, Science, and Technology of Japan during the conduct of the study. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Y. Kataoka: Resources, formal analysis, validation, investigation, visualization, methodology, writing-review and editing. M. Iimori: Conceptualization, resources,
formal analysis, funding acquisition, validation, investigation, visualization, methodology, writing review and editing. R. Fujisawa: Resources, formal analysis, validation, investigation, visualization, methodology. T. Morikawa-Ichinose: Resources, data curation, formal analysis, validation, investigation, methodology. S. Niimi: Resources, formal analysis, investigation, visualization, methodology. T. Wakasa: Validation. H. Saeke: Validation. E. Oku: Validation. D. Miura: Resources, data curation, software, formal analysis, supervision, validation, visualization, methodology. T. Tsurimoto: Resources, supervision, validation, methodology. Y. Maehara: Supervision, funding acquisition. H. Kito: Conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing original draft, project administration, writing review and editing.

Acknowledgments

We would like to thank Ms. Masako Kostogi, Tomomi Takada, Naoko Katakura, and Atsuko Yamaguchi for their expert technical assistance; Dr. Yoko Katsuki for technical advice; and Drs. Mamaro Kinawi and Minoru Takata for critical reading of the manuscript. We also appreciate the technical assistance from the Research Support Center, Research Center for Human Disease Modeling, Kyushu University Graduate School of Medical Sciences. This study was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to H. Kito, JSPS KAKENHI grant number 17H03598 and 18K19478) and by commercial grants of Shimohin Foundation of Advanced Medical Treatment Research (to M. Iimori).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 25, 2019; revised April 15, 2020; accepted May 21, 2020; published first May 28, 2020.

References


DRS and p53-Dependent Cell Fate Decision by FTD

Published OnlineFirst May 28, 2020; DOI: 10.1158/1541-7786.MCR-19-1051

Downloaded from mcr.aacrjournals.org on January 4, 2022. © 2020 American Association for Cancer Research.
50. Sabapathy K, Lane DP. Therapeutic targeting of p53: all mutants are equal, but some mutants are more equal than others. Nat Rev Clin Oncol 2018;15:13–30.