Trifluridine Induces p53-Dependent Sustained G2 Phase Arrest with Its Massive Misincorporation into DNA and Few DNA Strand Breaks

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Abstract

Trifluridine (FTD) is a key component of the novel oral antitumor drug TAS-102, which consists of FTD and a thymidine phosphorylase inhibitor. Like 5-fluoro-2'-deoxyuridine (FdUrd), a deoxynucleoside form of 5-fluorouracil metabolite, FTD is sequentially phosphorylated and not only inhibits thymidylate synthase activity, but is also incorporated into DNA. Although TAS-102 was effective for the treatment of refractory metastatic colorectal cancer in clinical trials, the mechanism of FTD-induced cytotoxicity is not completely understood. Here, we show that FTD as well as FdUrd induce transient phosphorylation of Chk1 at Ser345, and that this is followed by accumulation of p53 and p21 proteins in p53-proficient human cancer cell lines. In particular, FTD induced p53-dependent sustained arrest at G2 phase, which was associated with a proteasome-dependent decrease in the Cyclin B1 protein level and the suppression of CCNB1 and CDK1 gene expression. In addition, a p53-dependent increase in p21 protein was associated with an FTD-induced decrease in Cyclin B1 protein. Although numerous ssDNA and dsDNA breaks were induced by FdUrd, few DNA strand breaks were detected in FTD-treated HCT-116 cells despite massive FTD misincorporation into genomic DNA, suggesting that the antiproliferative effect of FTD is not due to the induction of DNA strand breaks. These distinctive effects of FTD provide insights into the cellular mechanism underlying its antitumor effect and may explain the clinical efficacy of TAS-102. Mol Cancer Ther; 14(4); 1004–13. ©2015 AACR.

Introduction

5-fluorouracil (5-FU) has long been a key drug of first-line treatment for a range of human cancers, particularly colorectal carcinoma. 5-FU–based chemotherapy has significantly improved and produced good therapeutic results in many patients, but some patients either acquire resistance or do not respond to 5-FU (1). 5-FU cytotoxicity has been linked to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme, thymidylate synthase (TS), by 5-fluoro-2'-deoxyuridine monophosphate (FdUMP; ref. 1). Despite the fact that the major cause of 5-FU cytotoxicity is its effect on RNA metabolism (2), 5-FU or 5-fluoro-2'-deoxyuridine (FdUrd), a deoxynucleoside form of the 5-FU metabolite, also affects DNA metabolism by inducing several types of DNA damage, especially during DNA replication (3). Consistently, 5-FU– or FdUrd-induced cytotoxicity is correlated with DNA damage during DNA replication (4, 5). DTTP starvation due to TS inhibition by FdUMP may perturb replication fork progression or replication-coupled DNA repair processes (3) and enhance misincorporation of dUTP or FdUTP into DNA, which becomes a target of uracil DNA glycosylases (6, 7). The repair intermediates are converted into DNA single- or double-strand breaks, which may then cause cytotoxicity (3, 7).

Trifluridine (FTD; also known as trifluoroorthymidine) is a fluorinated pyrimidine-type compound that is currently approved as an antiviral drug against herpes simplex virus (8). FTD is phosphorylated to a monophosphate form (FTD-MP), which inhibits TS (9, 10), and to a triphosphate form (FTD-TP), which is incorporated into DNA (11). When misincorporated FTD forms an FTD-G mismatch, it becomes a target of TGD or MBD4 (11). In support of the similar biochemical characteristics of FdUrd and FTD, FTD-resistant cell lines exhibit significant resistance to FdUrd and vice versa (12). TAS-102 is a novel oral nucleoside antitumor agent consisting of both FTD and tipiracil hydrochloride, a thymidine phosphorylase inhibitor (TPI) that inhibits catalytic degradation of FTD, at a molar ratio of 1:0.5 (13). Coadministration of TPI at this molar ratio potentiates the antitumor effect of FTD in vivo (14). Intriguingly, in a xenograft model, TAS-102 exhibits similar antitumor activity on FU-resistant cells and their parental cell line (15). In addition, TAS-102 has been developed for use in the clinic (16–19) and surprisingly, significantly improves overall...
survival of patients with metastatic colorectal cancer who were refractory or intolerant to fluoropyrimidine, irinotecan, or oxaliplatin in a placebo-controlled, double-blinded randomized phase II study (20). However, it is not fully understood how FTD exerts its cytotoxic effects on tumor cells that are resistant to chemotherapeutic drugs.

To identify the mechanism of FTD-induced cytotoxicity, we analyzed the DNA damage response following exposure to FTD or FdUrd in human cancer cell lines. We found that FTD or FdUrd activated similar DNA damage response pathways involving transient phosphorylation of Chk1 at Ser345 and accumulation of p53 and p21 proteins in p53-proficient human cancer cell lines. In particular, FTD induced p53-dependent sustained G2 phase arrest, which was associated with a proteasome-dependent decrease of Cyclin B1 and suppression of CCNB1 and CDK1 gene expression. However, although FdUrd induced numerous ssDNA and dsDNA breaks, FTD did not measurably induce DNA strand breaks. Instead, massive misincorporation of FTD into DNA was observed. These results suggest that the antiproliferative effect of FTD is not due to the induction of DNA strand breaks.

Materials and Methods

Cell culture and reagents

HCT-116 and RKO cells were purchased from the ATCC in 2011. AS49 cells were kindly provided by Dr. M. Takeshita (Kyushu University, Japan). These cells were authenticated by short tandem repeat analysis in 2014. HCT-116 p53−/− cells were kindly provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD), and were confirmed to be p53 null by immuno blot analysis (Fig. 2A), but otherwise were not authenticated. Cell lines were passaged to prepare low passage stocks, which were then cryopreserved in liquid nitrogen. Cell cultures were prepared from frozen stocks when the cells had undergone 2 months of continuous cultivation. Cells were cultured in DMEM supplemented with 10% FBS, 100 μ/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO2. siRNA transfection was performed using RNAiMax reagent (Invitrogen). The siRNA sequences used were: TP53 (5'-GAAAUUUGCGUGUGGAGUAUU-3'); CDK1 (5'-GGCGTACAGGGATAGCACAGC-3'); 0.002% Bromophenol Blue, 5% β-mercaptoethanol, 10% glycerol. Protein concentration was determined using the RC DC Protein Assay Kit (Bio-Rad) and equal amounts of protein (10 μg/lane) were subjected to SDS-PAGE. ImageQuant LAS 4000 Mini (GE Healthcare UK Ltd.) was used for detection and signal intensity was quantified by ImageJ software (NIH). The following antibodies were used: anti-Chk1 (#2360; Cell Signaling Technology (CST)), anti-Chk2 (#3440; CST), anti-Phospho-Chk1 (Ser345; #2341; CST), anti-Phospho-Chk2 (Thr68; #2661; CST), anti-TS (Taiho Pharmaceutical Co., Ltd.), anti-p53 (Clone DO-7; DakoCytomation), anti-p21 (C-19; Santa Cruz Biotechnology), anti-Cyclin B1 (clone G83; Millipore), anti-Cdk2/Cdk1 (PSTAIR; Millipore), anti-Phospho-Cdk1 (Tyr15; #9111; CST), and anti-β-actin (clone AC-74; Sigma). Statistical analysis in Fig. 5B was performed by the unpaired t test.

Cell viability assay

Alkaline or neutral comet assays were performed according to the manufacturer’s instructions (Trevigen). Following drug treatment, cells were collected, resuspended in ice-cold PBS at 106 cells/mL, mixed with low-melt agarose (1:10 ratio), and spread on frosted glass slides. For the alkaline-comet assay, the slides were successively submerged in lysis and alkaline solutions after the agarose had solidified. The slides were then subjected to electrophoresis (1 V/cm distance between electrodes) for 50 minutes at 24 V in alkaline solution under dark conditions. The cells were then fixed with 70% ethanol and stained with SYBR Green. For the neutral-comet assay, the cells were lysed without alkaline denaturation and subjected to electrophoresis (1 V/cm distance between electrodes) for 18 minutes at 24 V in TBE solution. The tail moment was determined using CometScore software (TriTek). Statistical analysis of Fig. 6B–E was performed by the Kruskal–Wallis test.

Quantitation of FdUrd and FTD incorporation into DNA

HCT-116 cells were cultured in the presence of 0.5 μM/L 14C-FdUrd or 5 μM/L 14C-FTD (Moravek Biochemicals) for the indicated times. Genomic DNA was purified using the QIAamp DNA Blood Mini Kit (QIAGEN). Radioactivity (dpm) was measured by liquid scintillation counting, and incorporation (pmol) of FdUrd or FTD into DNA was determined by measuring the amount of radioactivity per μg of DNA. The amount of radioactive analogues per μg of DNA was estimated on the basis of the relative activity of purified nucleoside analogues (14C-FdUrd: 8.5 fmol/dpm; 14C-FTD: 8.4 fmol/dpm).

RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA extraction and cDNA synthesis were performed using the RNeasy Mini Kit (QIAGEN) and the SuperScript III First-Strand Synthesis SuperMix (Invitrogen), respectively, according to the manufacturer’s instructions. Quantitative PCR was performed using the Quantifast SYBR Green PCR Kit (QIAGEN) using the following primers: CDK1: QTO0042672; CCNB1: QTO0006615 (QIAGEN); ACTB: forward (5'-CTGGCACCACACTTCTACATG-3'); reverse (5'-GGGTACGCGTGGATACGACGC-3'). mRNA levels were normalized against those of ACTB. Statistical analysis of Fig. 4B and E was performed by the Dunnett test.

Cell viability assay

Crystal violet staining was performed to measure cell viability. Briefly, cells were cultured with FTD or FdUrd in 96-well microplates. After treatment for 72 hours, cells were fixed with 4% paraformaldehyde for 20 minutes, stained with 0.05% crystal violet in 20% methanol for 20 minutes, and then rinsed in tap water. The plates were dried on paper for 1 hour and 100 μL of a 1:1 mixture of ethanol and 0.1 mol/L sodium phosphate was added to each well. The absorbance was measured at 570 nm by iMark (Bio-Rad). Cell viability is indicated as the percentage of the number of viable cells in cultures treated with FTD or FdUrd mock to the number of viable cells in cultures treated with solvent only.

Immunoblot analysis

Cell pellets were lysed in RIPA buffer (1.0% NP40, 50 mmol/L Tris HCl (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, 0.1% SDS) containing 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail (Nacalai Tesque) and incubated for 30 minutes on ice. The supernatant was cleared by centrifugation for 15 minutes at 15,000 × g and denatured by adding 1× SDS sample buffer [62.5 mmol/L Tris–HCl (pH 6.8), 2.5% SDS, 0.002% Bromophenol Blue, 5% β-mercaptoethanol, 10% glycerol]. Protein concentration was determined using the RC DC Protein Assay Kit (Bio-Rad) and equal amounts of protein (10 μg/lane) were subjected to SDS-PAGE. ImageQuant LAS 4000 Mini (GE Healthcare UK Ltd.) was used for detection and signal intensity was quantified by ImageJ software (NIH). The following antibodies were used: anti-Chk1 (#2360; Cell Signaling Technology (CST)), anti-Chk2 (#3440; CST), anti-Phospho-Chk1 (Ser345; #2341; CST), anti-Phospho-Chk2 (Thr68; #2661; CST), anti-TS (Taiho Pharmaceutical Co., Ltd.), anti-p53 (Clone DO-7; DakoCytomation), anti-p21 (C-19; Santa Cruz Biotechnology), anti-Cyclin B1 (clone G83; Millipore), anti-Cdk2/Cdk1 (PSTAIR; Millipore), anti-Phospho-Cdk1 (Tyr15; #9111; CST), and anti-β-actin (clone AC-74; Sigma). Statistical analysis in Fig. 5B was performed by the unpaired t test.
Cell-cycle analysis

HCT-116, HCT-116 p53−/−, RKO, and A549 cells cultured in the presence of FTD, FdUrd, or nocodazole were fixed with 70% ice-cold ethanol. To determine DNA content, cells were stained with 1 μg/mL propidium iodide (PI) in the presence of 0.5 μg/mL RNase A. To detect mitotic cells, cells were incubated with anti–phospho-Ser/Thr-Pro antibody, MPM-2 (Millipore), and then stained with Alexa488-conjugated anti-mouse IgG (Invitrogen) and PI. The samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Statistical analysis of Fig. 1C, 2C and 3B was performed by the paired t-test.

Immunofluorescent imaging

After cultivation of HCT-116 cells on coverslips and drug treatment, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% NP40, and blocked with 3% BSA. Immunostaining was performed with anti-Cyclin B1 antibody and Alexa488-conjugated anti-mouse IgG (Invitrogen) and PI. The samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Statistical analysis of Fig. 5D was performed by the unpaired t-test.

Results

FdUrd or FTD at IC50 concentrations induces accumulation of p53 and p21, and FTD induces sustained arrest at G2 phase

To compare cellular responses with FdUrd and FTD, human colon cancer HCT-116 cells were cultured in the presence of IC50 concentrations of FdUrd (0.5 μmol/L) or FTD (5 μmol/L). FdUrd induces phosphorylation of Chk1 at Ser345 (pS345 Chk1; ref. 22). Consistently, both FdUrd and FTD induced pS345 Chk1, which peaked at 12 hours (Fig. 1A). pS345 Chk1 levels then decreased gradually and the cells progressed into middle S phase semisynchronously at 36 hours for FdUrd and 24 hours for FTD, and finally accumulated with a 4N DNA content at 48 hours for FdUrd.
and 36 hours for FTD (Fig. 1B). When pS345 Chk1 was attenuated, the amount of TS protein increased, and phosphorylation of Chk2 at Thr68 (pT68 Chk2) and accumulation of p53 and p21 were observed (Fig. 1A). Intriguingly, the accumulation of cells with a 4N DNA content was maintained, but was not associated with mitotic chromosomal condensation (the mitotic index, which was evaluated by the proportion of MPM-2$^{+}$ cells, Fig. 1C), suggesting sustained arrest at G2 phase. Similar cellular responses to IC50 concentrations of FdUrd or FTD were observed in the other colon cancer cell line RKO (Supplementary Fig. S1A) and the lung adenocarcinoma cell line A549 (Supplementary Fig. S2A). However, sustained G2 arrest was only observed in FTD-treated cells (Supplementary Figs. S1B and S2B).

To examine whether mitotic entry is inhibited by FTD treatment, mitosis in HCT-116 cells in the presence or absence of 5 μmol/L FTD was monitored when mitotic progression was blocked by nocodazole, an inhibitor of microtubule polymerization. Less than 10% of HCT-116 cells became MPM-2$^{+}$ in the absence of FTD (Fig. 1D). Similar phenomena were observed in A549 cells (Supplementary Fig. S2D), indicating that FTD prevented cells from proceeding into mitosis.

**FTD-induced inhibition of mitotic entry is p53 dependent**

The sustained accumulation of cells with a 4N DNA content could be a primary reason for the growth-suppressive effect of FTD. Because the accumulation of p53 and p21 proteins was induced during this arrest, we tested whether p53 is involved in this process. First, we determined the IC50 concentrations of FdUrd and FTD in HCT-116 p53−/− cells as 10 μmol/L (FdUrd) and 5 μmol/L (FTD), respectively. pS345 Chk1 was induced in HCT-116 p53−/− cells cultured in the presence of FdUrd (10 μmol/L) or FTD (5 μmol/L; Fig. 2A). The pS345 Chk1 level decreased at 8 hours (Fig. 2A) and consistently, HCT-116 p53−/− cells proceeded to middle S phase earlier than HCT-116 cells (at 12 hours for FdUrd and 8 hours for FTD; Fig. 2B), as the amount of TS protein increased (Fig. 2A). However,
sustained accumulation of cells with a 4N DNA content (Fig. 2B) and lower numbers of MPM-2” cells (Fig. 2C) were not observed. Rather, the MPM-2” cell population increased when HCT-116 p53-/- cells were treated with FTD (Fig. 2C). These results suggest that FTD-induced arrest at G2 phase was dependent on functional p53.

Next, we monitored the accumulation of mitotic cells in the presence of nocodazole in HCT-116 p53-/- cells. More than 40% of cells became MPM-2” in both the presence and absence of FTD (Fig. 2D), suggesting that p53 is required to block mitotic entry in the presence of FTD.

To further confirm that the loss of p53 causes abrogation of sustained arrest at G2 phase by FdUrd or FTD, we depleted HCT-116 cells of p53 by using siRNA, cultured them in the presence of FdUrd (0.5 μmol/L) or FTD (5 μmol/L) for 72 hours, and analyzed their cell-cycle status and mitotic index. siRNA-mediated depletion of p53 protein was confirmed in FdUrd- or FTD-treated cells (Fig. 3A). FdUrd- or FTD-induced accumulation of p21 protein was also suppressed, indicating that p53 activity had been almost completely lost (Fig. 3A). Under these conditions, although the number of MPM-2” cells was significantly decreased by FdUrd or FTD in HCT-116 cells treated with control (GL2) siRNA, no decrease was observed in HCT-116 cells treated with TP53 siRNA (Fig. 3B). Similar results were obtained in the other human cancer cell line A549 (Supplementary Fig. S3). Collectively, these results indicate that p53 plays a critical role in the induction of sustained arrest at G2 phase by FTD via the suppression of mitotic entry.

FTD decreases Cyclin B1 and Cdk1 proteins in a p53-dependent manner

Next, we examined the downstream mediators of p53-dependent G2 phase arrest. Cdk1, the cyclin-dependent kinase that together with Cyclin B1 normally drives cells into mitosis, is the ultimate target of pathways that mediate arrest at G2 phase in response to DNA damage (23–25). Cdk1 kinase activity is also negatively regulated by inhibitory phosphorylation of Tyr15 (pY15) by Wee1 (26). Treatment with FdUrd (0.5 μmol/L) or FTD (5 μmol/L) decreased Cyclin B1 protein and intriguingly, also suppressed pY15 Cdk1 in GL2 siRNA-treated HCT-116 cells at 72 hours (Fig. 3A). Cdk1 protein was also lower in FTD-treated cells (Fig. 3A). By contrast, neither a decrease in Cyclin B1 or Cdk1 protein levels nor suppression of pY15 Cdk1 was observed in TP53 siRNA-treated HCT-116 cells (Fig. 3A). A p53-dependent decrease of Cyclin B1 and Cdk1 protein levels was observed when A549 cells were treated with FTD (4 μmol/L; Supplementary Fig. S3A). These results indicate that p53 is required for the FTD-induced sustained arrest at G2 phase, which is possibly mediated by the decrease in the level of Cyclin B1 or Cdk1 protein. The contribution of inhibitory phosphorylation of Cdk1 (pY15 Cdk1) to p53-dependent sustained G2 arrest by FTD should be minor, because FTD clearly suppressed pY15 Cdk1 in GL2 siRNA-treated cells (Fig. 3A and Supplementary Fig. S3A).

FTD suppresses the transcription of CCNB1 and CDK1 and promotes the proteasome-dependent decrease of Cyclin B1

Next, we asked whether the FTD-induced decrease of Cyclin B1 and Cdk1 protein levels occurred at the level of transcriptional regulation. First, we examined the time course of the Cyclin B1 and Cdk1 protein levels in HCT-116 cells. When HCT-116 cells were cultured in the presence of FdUrd or FTD, transient increases in Cyclin B1, Cdk1, and pY15 Cdk1 protein levels were observed from 12 to 36 hours (Fig. 4A). At 48 hours, the amount of Cyclin B1 protein and pY15 Cdk1 was dramatically decreased (Fig. 4A) and at 72 hours, the amount of Cdk1 protein was also decreased (Fig. 4A). Similar results were obtained when RKO and A549 cells were used (Supplementary Fig. S4). The change in the amount of Cyclin B1 protein was further confirmed as FTD-induced transient increase and the following decrease of cells with Cyclin B1-positive staining by immunofluorescent imaging (Supplementary Fig. S5C and S5D). At the transcriptional level, however, significant suppression of CCNB1 and CDK1 mRNA expression was observed in both FdUrd- and FTD-treated cells at 72 hours, but only slight suppression of CCNB1 mRNA expression by FTD was observed at 48 hours (Fig. 4B). We then examined whether or not the proteasome was involved in the Cyclin B1 decrease observed from 36 to 48 hours in FTD-treated HCT-116 cells. When we added the proteasome inhibitor MG132 (1 μmol/L) at 36 hours,
the FTD-induced Cyclin B1 protein decrease from 36 to 48 hours was partially alleviated, whereas decreases in the pY15 Cdk1 level or the Cdk1 protein level was not affected (Fig. 4C). The FTD-induced decrease in the number of cells with cytoplasm dominant Cyclin B1 was also alleviated by MG132 (Supplementary Fig. S6A and S6B). By contrast, when HCT-116 p53<sup>−/−</sup> cells were cultured in the presence of FdUrd or FTD, neither the amount of Cyclin B1 or Cdk1 proteins nor the pY15 Cdk1 level changed significantly (Fig. 4D). In addition, no change in CCNB1 or CDK1 mRNA expression was observed (Fig. 4E). These results indicate that p53 is involved both in proteasomal degradation of Cyclin B1 protein and in the transcriptional suppression of gene expression of mitotic regulators, which would contribute to FTD-induced sustained G2 phase arrest.

FTD-induced decrease of Cyclin B1 protein is dependent on p21

In response to DNA damage, p21 mediates Cyclin B1 degradation, which is blocked by MG132 in HCT-116 cells (27). Because FTD induced p21 accumulation (Figs. 1A, 3A; Supplementary Figs. S1A and S2A), we examined whether p21 was involved in the FTD-induced Cyclin B1 protein decrease. We confirmed CDKN1A (gene symbol of p21) siRNA-mediated suppression of p21 expression. The p53-dependent decrease of Cyclin B1 and Cdk1 proteins and mRNA by FdUrd and FTD. A, Western blot analysis. HCT-116 cells were cultured in the presence of 0.5 μmol/L FdUrd or 5 μmol/L FTD for indicated time. B, quantitation of CCNB1 and CDK1 mRNA; ***, P < 0.001 and **, P < 0.01. C, top, timeline for exposure to FTD and MG132. Bottom, Western blot analysis. HCT-116 p53<sup>−/−</sup> cells were cultured in the presence of 10 μmol/L FdUrd or 5 μmol/L FTD for indicated times. E, quantitation of CCNB1 and CDK1 mRNA; error bars, the SD of three independent experiments.
accumulation, but not of p53 accumulation (Fig. 5A). The Cyclin B1 protein decrease induced by FTD was observed from 24 to 48 hours in cells treated with GL2 siRNA, but the decrease was partially alleviated in cells treated with CDKN1A siRNA (Fig. 5A). The difference in Cyclin B1 protein level at 48 hours, but not at 0 or 24 hours, between GL2 and CDKN1A siRNA-treated cells was statistically significant (Fig. 5B). Consistently, the FTD-induced decrease in the number of cells with cytoplasm dominant Cyclin B1 from 24 to 48 hours was also partially alleviated in cells treated with CDKN1A siRNA (Fig. 5C and D). These results indicate that p21 is involved in the FTD-induced decrease of Cyclin B1 protein.
FTD is efficiently incorporated into genomic DNA in a time-dependent manner

FdUrd and FTD are incorporated into genomic DNA during replication (11). To determine how much of these two nucleoside analogues were incorporated into genomic DNA during proliferation, we cultured HCT-116 cells in the presence of 14C-radiolabeled FdUrd or FTD. Genomic DNA was then purified from cells at different time points and the amount of radioactivity was measured by scintillation counting. The amount of incorporated FdUrd or FTD was estimated by radioactivity/mole of purified 14C-labeled FdUrd or FTD. The lines are median and the bars indicate inter-quartile range; **, P < 0.01.
damage responses in human cancer cell lines (Fig. 1A; Supplementary Figs. S1B and S1C, and S2B) and mitosis. Recent reports showed that transient activation of p53 at contrast with FdUrd, FTD did not induce any apparent DNA damage. Because FTD is a fluorinated thymidine analogue that exerts an inhibitory effect on TS activity in its monophosphate form (9, 10), and D; Supplementary Figs. S1B and S1C, and S2B) did not occur in the absence of p53, FTD might induce cell death after allowing p53-deficient cells to enter mitosis. Further analysis is necessary to clarify these possibilities.

Our experiment using radiolabeled FdUrd and FTD revealed that FTD was incorporated into genomic DNA far more efficiently than FdUrd (Fig. 6A), which is consistent with a previous report using HeLa cells (11). FdUrd increases the amount of FdUTP, but this is quickly dephosphorylated to FdUMP by the highly active cellular enzyme dUTPase to prevent its misincorporation into genomic DNA (33). By contrast, FTD increases the amount of FTD incorporation into FdUMP (i.e., TAS-102; refs. 13, 34) could result in DNA fragmentation detectable by alkaline gel electrophoresis, in situ nick translation (13), or pulse field gel electrophoresis under the neutral condition (34).

Our cellular analysis of the p53-proficient human cancer cell lines revealed that the two different fluoropyrimidine-type drugs, FdUrd and FTD, activated similar DNA damage response pathways with different cellular outcomes. In contrast with FdUrd and many other chemotherapeutic drugs used for standard cancer treatment, FTD induced DNA strand breaks with its massive misincorporation into DNA. This distinctive activity of FTD may provide insights how FTD elicits its antitumor effect and open ways to increase the clinical efficacy of TAS-102.

FDiRd, but not FTD, induces single- and double-strand breaks in genomic DNA

Next, we evaluated DNA strand breaks that could be induced by these compounds. HCT-116 cells were cultured in the presence of FdUrd or FTD at IC50 concentrations for 24 and 72 hours, and ssDNA and dsDNA breaks were evaluated by alkaline- and neutral-comet assays. As reported previously (28, 29), a significant increase in the mean score of the tail moment was observed in alkaline- and neutral-comet assays after camptothecin treatment (Supplementary Fig. S7). When cells were cultured in the presence of FdUrd, a drastic increase in the mean score of the tail moment was observed in the alkaline-comet assay at both 24 (Fig. 6B) and 72 hours (Fig. 6D). A significant increase was also observed in the neutral-comet assay at 24 (Fig. 6C) and 72 hours (Fig. 6E). By contrast, when cells were cultured in the presence of FTD, no significant difference in the mean score of the tail moment was observed in either the alkaline-comet assay (Fig. 6B and D) or the neutral-comet assay (Fig. 6C and E). These results indicate that FdUrd induces extensive DNA ssDNA and dsDNA breaks, but that FTD does not induce any measurable DNA strand breaks, although it is efficiently incorporated into genomic DNA.

Discussion

FTD is a fluorinated thymidine analogue that exerts an inhibitory effect on TS activity in its monophosphate form (9, 10), and is incorporated into DNA in its triphosphate form (11). In this study, we found that FTD and FdUrd induced similar DNA damage responses in human cancer cell lines (Fig. 1A; Supplementary Figs. S1A and S2A). FTD induced p53-dependent sustained arrest at G2 phase (Fig. 1B; Supplementary Figs. S1B and S2B), which was associated with a proteasome-dependent decrease in the Cdk1 protein level (Fig. 4C) and transcriptional suppression of CCNB1 and Cdk1 genes (Fig. 4B). In contrast with FdUrd, FTD did not induce any apparent DNA strand breaks (Fig. 6B–E), despite its efficient misincorporation into genomic DNA (Fig. 6A).

It is intriguing that, following the transient induction of p53, the decrease of Cyclin B1 and Cdk1 proteins at G2 phase would reversibly withdraw cells from the cell cycle before mitosis. Recent reports showed that transient activation of p53 at G2 phase decreases the levels of mitotic regulators before mitosis and is sufficient to induce permanent cell-cycle withdrawal, also called senescence, in normal human diploid fibroblasts (30, 31). During senescence induction, Cyclin B1 is translocated into the nucleus and degraded by the proteasome through premature activation of APC/C•Cdh1 at G2 phase in a p53- and p21-dependent manner (30, 31). In addition, transcriptional suppression of CCNB1 has also been observed to occur during senescence induction in a p53 and pRb family protein-dependent manner (30). Because FTD decreased the Cyclin B1 protein level in a p53−/− (Figs. 3A and 4A and D), p21−/− (Fig. 5), and proteasome (Fig. 4C)−/−-dependent manner and suppressed the transcription of CCNB1 and CDK1 genes in a p53-dependent manner (Fig. 4B and E), FTD might have potential to induce a “senescence-like” status even in p53-proficient human tumor cells. Conversely, FTD exerts similar cytotoxic effects regardless of p53 status (this work; ref. 32). Because the downregulation of mitotic regulators (Figs. 3A and 4D and E and Supplementary Fig. S3A) or the inhibition of mitotic entry (Figs. 2C and D and 3B and Supplementary Fig. S3B) did not occur in the absence of p53, FTD might induce cell death after allowing p53-deficient cells to enter mitosis. Further analysis is necessary to clarify these possibilities.

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Disclosure of Potential Conflicts of Interest

M. Iimori, S. Kiyonari, K. Ando, E. Tokunaga, Y. Maehara, and H. Kitao report receiving commercial research grants from Taiho Pharmaceutical Co. Ltd. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.); K. Matsuoka, S. Niimi, H. Tsukihara, H. Kitao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis); K. Matsuoka, M. Iimori, S. Niimi, H. Tsukihara, S. Watanabe, S. Kiyonari, M. Kiniwa, K. Ando, E. Tokunaga, Y. Maehara, H. Kitao
Writing, review, and/or revision of the manuscript; K. Matsuoka, M. Iimori, S. Watanabe, H. Kitao
Administrative, technical, or material support (i.e., reporting or organizing
Writing, review, and/or revision of the manuscript: M. Kiniwa, E. Oki, H. Kitao
Other (data discussion): H. Saeki

Acknowledgments
The authors thank Chie Iwamoto, Naoko Katakura, Akiko Sea, Haruna Masuda, Tomomi Takada, and Maiko Iseki for their expert technical assistance. The authors also appreciate the technical assistance from the Research Support Center, Research Center for Human Disease Modeling, Kyushu University Graduate School of Medical Sciences.

References

Molecular Cancer Therapeutics

Trifluridine Induces p53-Dependent Sustained G2 Phase Arrest with Its Massive Misincorporation into DNA and Few DNA Strand Breaks

Kazuaki Matsuoka, Makoto Iimori, Shinichiro Niimi, et al.

Mol Cancer Ther 2015;14:1004-1013. Published OnlineFirst February 19, 2015.

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doi:10.1158/1535-7163.MCT-14-0236

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