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DNA Damage and Repair

Molecular Cancer Research

Thymidine Kinase 1 Loss Confers Trifluridine Resistance without Affecting 5-Fluorouracil Metabolism and Cytotoxicity

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Abstract

Acquired resistance to therapeutic drugs is a serious problem for patients with cancer receiving systemic treatment. Experimentally, drug resistance is established in cell lines *in vitro* by repeated, continuous exposure to escalating concentrations of the drug; however, the precise mechanism underlying the acquired resistance is not always known. Here, it is demonstrated that the human colorectal cancer cell line DLD1 with acquired resistance to trifluridine (FTD), a key component of the novel, orally administered nucleoside analogue-type chemotherapeutic drug trifluridine/tipiracil, lacks functional thymidine kinase 1 (TK1) expression because of one nonsense mutation in the coding exon. Targeted disruption of the *TK1* gene also conferred severe FTD resistance, indicating that the loss of TK1 protein expression is the primary cause of FTD resistance. Both FTD-resistant DLD1 cells and DLD1-*TK1*$^{-/-}$ cells exhibited similar 5-fluorouracil (5-FU) sensitivity to that of the parental DLD1 line. The quantity of cellular pyrimidine nucleotides in these cells and the kinetics of thymidylate synthase ternary complex formation in 5-FU–treated cells is similar to DLD1 cells, indicating that 5-FU metabolism and cytotoxicity were unaffected. The current data provide molecular-based evidence that acquired resistance to FTD does not confer 5-FU resistance, implying that 5-FU–based chemotherapy would be effective even in tumors that become refractory to FTD during trifluridine/tipiracil treatment. *Mol Cancer Res; 16(10); 1483–90. ©2018 AACR.*

Introduction

Systemic therapy is a prevalent method for the treatment of cancer; however, the acquisition of resistance to a therapeutic drug during long-term treatment can be a devastating problem. In such cases, the identification of alternative regimens is essential. Trifluridine/tipiracil (TFTD; formerly known as TAS-102) is a novel, orally administered anticancer drug. In placebo-controlled clinical trials, TFTD significantly improved the overall survival and progression-free survival of patients with metastatic colorectal cancer who were refractory to prior chemotherapy regimens including 5-fluorouracil (5-FU) or its prodrugs, oxaliplatin and irinotecan (1, 2). Trifluridine (FTD) is a fluorinated nucleoside analogue and a cytotoxic component of TFTD, and tipiracil hydrochloride is a thymidine phosphorylase (TP) inhibitor that suppresses the degradation of FTD *in vivo* and maintains the FTD concentration in the bloodstream (3). Once FTD is transported into the cytoplasm of tumor cells by nucleoside transporters, such as equilibrative nucleoside transporter 1 (hENT1) or hENT2 (4–6), it is phosphorylated to monophosphate (FTD-MP), diphosphate, and triphosphate (FTD-TP) forms by thymidine kinase (TK), thymidylate kinase (TYMK), and nucleoside diphosphate kinase (NDK), respectively, exerting cytotoxic effects via incorporation into DNA (7–9). In addition, FTD-MP inhibits thymidylate synthase (TS; refs. 10, 11) and may affect *de novo* dTTP biosynthesis. Thus, the mechanism underlying the antitumor effect of FTD differs from, but partially overlaps with, that of 5-FU. Consistently, in experimental settings, human cancer cell lines that acquire resistance to 5-FU do not exhibit resistance to FTD (12) or TFTD (13).

Several human cancer cell lines with acquired resistance to FTD have been established by repeated, continuous exposure of cells in culture to escalating concentrations of FTD (12, 14). Murakami and colleagues established an FTD-resistant line from the human colorectal adenocarcinoma cell line DLD1 (named DLD1-FTD), which has significantly lower TK activity than the parental line (12). FTD-resistant H630 colon cancer cells through intermittent exposure of FTD also decreased TK protein expression and activity, but FTD-resistant H630 cells through continuous exposure of FTD decreased hENT and increased secretory phospholipase-A2 (sPLA2) gene expression (4). A different approach using random mutagenesis and drug selection showed that loss of TK or TS activity in the mouse mammary carcinoma cell line FM3A is associated with severe FTD resistance (11). On the other hand,

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FTD-resistant DLD1 cells established by Tsunekuni and colleagues overexpress the miRNA *let-7d-5p*, which may be the cause of FTD resistance (14). In these cases, the FTD-resistant cells remain comparably sensitive to 5-FU. However, the molecular mechanism underlying the differential sensitivity to FTD and 5-FU remains to be fully elucidated.

The current study explored the decrease in TK activity in DLD1-FTD cells and its association with FTD resistance, as well as the differential sensitivity of FTD-resistant cells to 5-FU. Our results indicate that DLD1-FTD cells lack functional TK1 protein expression because of a nonsense mutation in the coding exon. Targeted disruption of the *TK1* gene using the CRISPR/Cas9 system (DLD1-*TK1*$^{-/-}$) conferred severe FTD resistance, confirming that loss of TK1 protein expression itself causes severe FTD resistance. The 5-FU sensitivity of both DLD1-FTD cells and DLD1-*TK1*$^{-/-}$ cells was similar to that of the parental line, and the levels of pyrimidine nucleotides of the 5-FU metabolic pathway and the kinetics of TS ternary complex formation were not altered, indicating that 5-FU metabolism and cytotoxicity were unaffected. The current data support the notion that 5-FU and its prodrugs remain effective even in tumors with acquired resistance to FTD.

Materials and Methods

Cell culture and reagents

The human colorectal cancer cell line DLD1 and its FTD-, 5-FU-, and 5-fluoro-2′-deoxyuridine (FdUrd)–resistant derivative lines (referred to as DLD1-FTD, DLD1-FU, and DLD1-FdUrd, respectively) were provided by Taiho Pharmaceutical Co. Ltd. on April 8, 2009 (DLD1 and DLD1-FTD) and on July 14, 2015 (DLD1, DLD1-FU, and DLD1-FdUrd; ref. 12). These cells were authenticated by short tandem repeats analysis (Biologica Co.) on December 2, 2017, and confirmed negative for *Mycoplasma* infection with the MycoAlert Mycoplasma Detection Kit (Lonza) on March 22, 2018. DLD1-FTD cells stably expressing TK1 and *TK1* gene knockout DLD1 cells were generated as described in Supplementary Information. Cells were cultured in RPMI1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO_2. 5-FU, FdUrd, and FTD were purchased from Tokyo Chemical Industry.

Direct sequencing

The cDNA synthesis was performed using SuperScript III First-Strand Synthesis SuperMix with oligo-dT primer (Thermo Fisher Scientific). The *TK1* gene was amplified from cDNA from DLD1 and DLD1-FTD cells using the primer sets forward (5′-CACCATGAGCTGCATTAACCTGCCC-3′) and reverse (5′-TCAGTTGGCAGGGCTGCATTGCAG-3′). PCR amplification of the *TK1* genome region including exon 7 and direct sequencing were performed using the following primers: g*TK1*_forward, 5′-TCGGCACAGAGAAGGAGGTAGCTCCACC-3′; g*TK1*_reverse, 5′-AGCGTCCAGTAGGCGGCAGTGGCAG-3′. Direct sequencing of the PCR amplification product was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

Immunoblotting

Immunoblot analysis was performed as described previously (8). The following primary antibodies were used: anti-TK1 (ab57757; Abcam), anti-TS (ab108995; Abcam), and anti–β-actin (A5316; Sigma). Band intensity was quantified using ImageJ (NIH, Bethesda, MD).

Immunofluorescence imaging

DLD1 cells and their derivatives were cultured in the presence of various concentrations of FTD for 1 hour in black μClear 96-well microplates (Greiner Bio-One) and then fixed with 70% ethanol. The fixed samples were acid depurinated with 1.5 N HCl, blocked with 5% goat serum and 0.3% Triton X-100, prepared in PBS, incubated with anti-bromodeoxyuridine (BrdUrd) antibody (clone 3D4, BD Biosciences) diluted in PBS containing 1% BSA, immunostained with Alexa Fluor 488–conjugated secondary antibodies diluted in PBS containing 1% BSA, and stained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI). The immunostained plate was scanned using a Cytell system (GE Healthcare). Quantitative analysis of Alexa Fluor 488 fluorescence intensity in the DAPI-stained nuclei was performed using the IN Cell Investigator software (GE Healthcare) as described previously (6). Briefly, the Alexa Fluor 488 fluorescence intensity in each individual cell was measured, and the average in each well was calculated. Bar graphs indicate the relative scores calculated by defining the scores of maximum Alexa Fluor 488 fluorescence intensity value in each experiment as 1.

Quantification of pyrimidine nucleotides by LC-QqQ-MS

Intracellular UMP, UDP, UTP, and CTP were quantified using liquid chromatography/triple-stage quadrupole mass spectrometry (LC-QqQ-MS) as described previously (15). All metabolites were detected with optimized selective reaction monitoring transitions in negative ionization mode as follows [precursor ion (*m/z*)/product ion (*m/z*) scores are shown]: UMP: 322.7/78.85, 322.7/96.95, 322.7/110.9; UDP: 403/78.95, 403/158.8, 403/111; UTP: 482.6/384.85; CTP: 482/158.9. The quantity of metabolites was normalized by the amount of L(+)-10-camphor sulfonic acid (10CS), which was included in the quenching buffer (ice-cold 100% methanol). All chemicals were purchased from Sigma.

Statistical analysis

Statistical analysis was performed using JMP Pro 13 software (SAS Institute Inc.). The Student *t* test was performed on results shown in Fig. 4D; Supplementary Fig. S2D.

Results

Loss of functional TK1 protein in DLD1-FTD cells due to a point mutation in *TK1* gene

To explore the differences between DLD1-FTD cells and the parental DLD1 cells, we performed a comprehensive search of RNA sequencing (RNA-seq) data of DLD1-FTD cells and the parental DLD1 cell line to identify transcript variants [the NGS dataset from RNA-seq analysis was deposited in the Sequence Read Archives (SRP135850)]. We first focused on the genes that are categorized as "pyrimidine metabolism" in the Kyoto Encyclopedia of Genes and Genomes (Supplementary Table S1). Among 97 genes, we identified 5 genes (*TK1*, *DPYD*, *NT5C1B*, *UPB1*, and *CDA*) showing more than 2-fold expression level changes in DLD1-FTD cells. *TK1* was the only gene whose expression level has decreased more than 8-fold. In addition, *TK1* was the only gene with a homozygous and nonsynonymous mutation within its coding sequence that was only present in DLD1-FTD cells. Furthermore, the mutation found in the *TK1* gene produced a stop codon and caused prematurely

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truncation of the TK1 protein (c.740G>T(G177*), which would lack the essential catalytic domain of TK1 within its C terminus.

TK activity is significantly lower in DLD1-FTD cells than in the parent DLD1 cell line (12). We confirmed the nucleotide substitution within the protein encoding region of the *TK1* mRNA in DLD1-FTD cells by direct sequencing of cDNA (Fig. 1A). In addition, the nucleotide substitution in exon 7 of the *TK1* gene was confirmed using genomic DNA from DLD1-FTD cells (Fig. 1B). Next, we examined the TK1 protein expression by immunoblot analysis. The anti-TK1 antibody against the N-terminus (aa. 1–235) of the TK1 protein could not detect TK1 in DLD1-FTD cells (Fig. 1C). In contrast, no significant differences were observed between DLD1-FTD cells and the parental DLD1 cells in the expression of proteins involved in pyrimidine biosynthesis, including TS, dihydrofolate reductase (DHFR), ribonucleotide reductase M2 (RRM2), and dUTPase (DUT; Supplementary Fig. S1A). TP protein expression was not detected in DLD1 and DLD1-FTD cells (Supplementary Fig. S1B), and *TYMP* mRNA expression was not detected in DLD1 cells (Supplementary Fig. S1C). Furthermore, no significant down-regulation of *SLC29A1* or *SLC29A2* mRNA, which encode hENT1 and hENT2, respectively, was observed in DLD1-FTD cells (Supplementary Fig. S1D). A decrease of *sPLA2* mRNA expression was observed in DLD1-FTD cells (Supplementary Fig. S1D), although it should not contribute to FTD resistance because the overexpression of sPLA2 causes FTD resistance (4). These results indicate that DLD1-FTD cells lost functional TK1 protein expression during the development of FTD resistance.

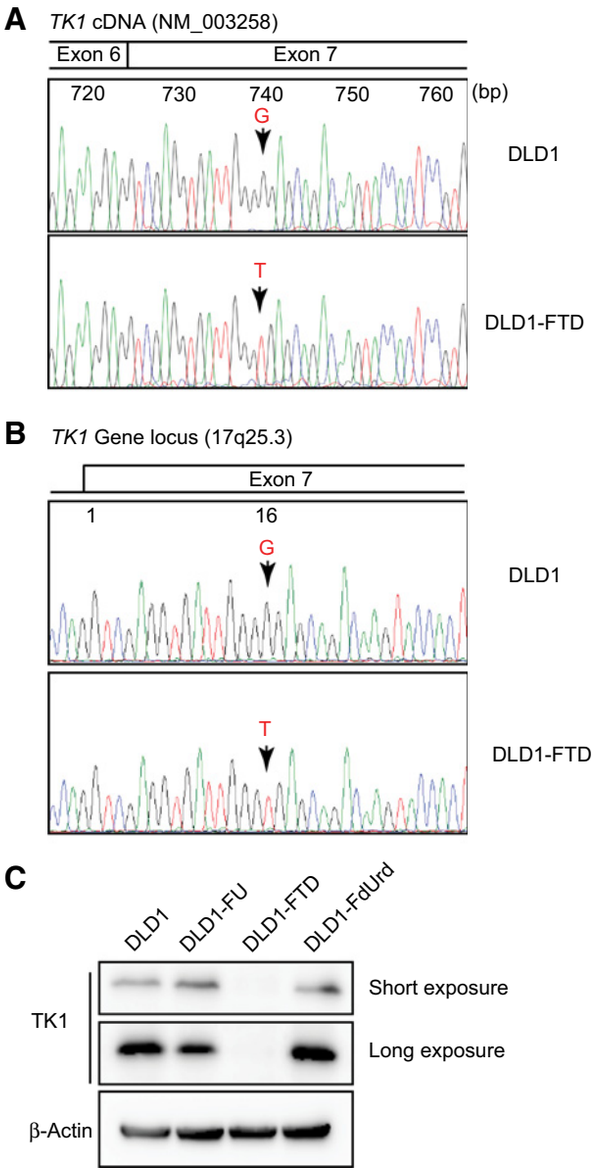

Figure 1.
Loss of TK1 protein expression in the DLD1 cells with acquired resistance to FTD (DLD1-FTD). Sequence chromatographs of *TK1* mRNA (**A**) and the *TK1* gene (**B**) in DLD1 and DLD1-FTD cells. The positions of the c.740G>T(G177*) mutation are indicated by arrows. **C,** TK1 protein expression in DLD1, the 5-FU–resistant (DLD1-FU), DLD1-FTD, and the FdUrd-resistant (DLD1-FdUrd) cells. Images represent long- and short exposure of TK1 immunoblots.

Reintroduction of TK1 restores FTD sensitivity in DLD1-FTD cells

TK is a critical enzyme for the activation of FTD inside the cell, and FM3A cells lacking TK activity are less sensitive to FTD than the parental line (11). To test whether loss of TK1 expression in DLD1-FTD cells is the primary cause of FTD resistance, we generated DLD1-FTD cells ectopically expressing TK1. Several DLD1-FTD cell lines stably expressing various levels of TK1 were obtained (Fig. 2A), among which two clones were selected for further analysis, one expressing an equal level (#19) and one expressing a 2-fold higher level (#13) of the TK1 protein compared with the endogenous TK1 protein level in the parental DLD1 cells. The IC_{50} values of FTD in DLD1-FTD/TK1#13 and DLD1-FTD/TK1#19 cells were comparable with that in DLD1 cells, while far more sensitive than that in DLD1-FTD cells (Table 1), indicating that ectopic expression of the TK1 protein restored FTD sensitivity in DLD1-FTD cells to the level of the parental line.

FTD cytotoxicity is significantly correlated with the degree of FTD incorporation into the DNA of cells (9, 16). To test whether ectopic expression of the TK1 protein affects the efficacy of FTD incorporation, each cell line was exposed to 0.1, 1, and 10 μmol/L FTD for 1 hour, and FTD incorporation was evaluated by immunofluorescent staining using an anti-BrdUrd antibody as described previously (6). Compared with DLD1 cells, which incorporated FTD in a dose-dependent manner, DLD1-FTD cells barely incorporated FTD even at a high concentration (10 μmol/L; Fig. 2B and C). DLD1-FTD/TK1#13 and DLD1-FTD/TK1#19 cells showed dose-dependent FTD incorporation into the DNA (Fig. 2C). These results indicate that loss of TK1 expression in DLD1-FTD cells is sufficient to cause severe resistance to FTD, possibly via a mechanism involving the inability to incorporate FTD into the DNA.

TK1 gene knockout by CRISPR/Cas9 renders DLD1 cells resistant to FTD

To confirm that loss of TK1 expression was the primary cause of FTD resistance, we first performed siRNA-mediated gene knockdown experiments (Supplementary Fig. S2A). Under the most stringent siRNA condition, in which siRNA transfection

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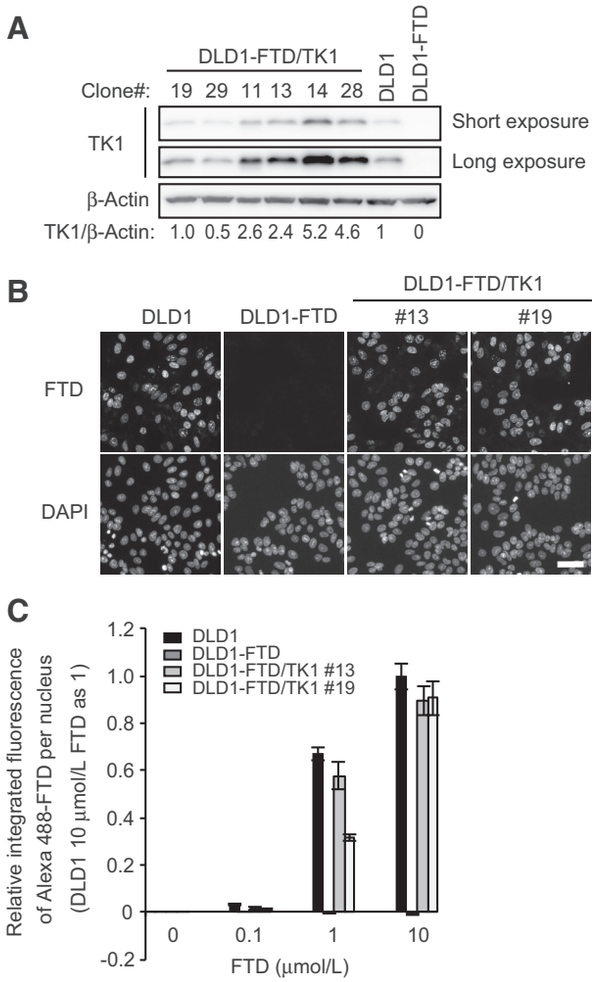

Figure 2.

Ectopic expression of the TK1 protein rescues the defect in the DLD1-FTD cell. **A,** TK1 protein expression in six DLD1-FTD clones stably expressing TK1 (DLD1-FTD/TK1). Images represent long- and short exposure of TK1 immunoblots are shown. **B,** Fluorescent immunostaining of FTD incorporated into DNA detected as detected by the anti-BrdUrd antibody. Nuclei were stained with DAPI. Representative images of each cell line exposed to 10 μmol/L FTD for 1 hour are shown. Scale bar, 50 μm. **C,** Relative integrated fluorescence of the FTD signal per nucleus. Bar graphs indicate the average of the relative scores of the integrated fluorescence intensity in each individual cell when the average score of DLD1 cells cultured in the presence of 10 μmol/L FTD for 1 hour is defined as 1. Error bars represent the SD of four independent experiments.

suppressed TK1 protein expression even after 120 hours (si*TK1*#1, 40 pmol; Supplementary Fig. S2B), the FTD IC_{50} value in these cells was only twice the value for that in DLD1 cells treated with si*Luc* (Supplementary Table S1). These cells were considerably more sensitive than DLD1-FTD cells (Supplementary Table S1). Consistent with these findings, si*TK1*-treated DLD1 cells showed dose-dependent FTD incorporation into DNA, although the fluorescence signal of the incorporated FTD was significantly lower in si*TK1*#1-treated DLD1 cells than in si*Luc*-treated DLD1 cells (Supplementary Fig. S2C and S2D).

Two possible explanations are proposed: One is the presence of redundant factors, such as TK2, which alleviate FTD resistance,

Table 1. TK1 protein expression and IC_{50} concentration of FTD

Cell lines	IC_{50} FTD (μmol/L)	TK1/β-actin
DLD1	3.95 ± 0.66	1
DLD1-FTD	>300	N.D.
DLD1-FTD/TK1 #13	5.56 ± 0.36	2.4
DLD1-FTD/TK1 #19	4.31 ± 1.00	1.0

Abbreviation: N.D., not determined.

and the other is that the residual expression of the TK1 protein in si*TK1*-treated cells confers FTD sensitivity. To test these possibilities, we first evaluated FTD incorporation into DNA in DLD1 cells treated with siRNA against *TK2* gene (Supplementary Fig. S2E). Similar levels of FTD incorporation into DNA were observed in the si*Luc* and si*TK2*-treated DLD1 cells (Supplementary Fig. S2F), indicating that TK2 does not play a crucial role in FTD activation and possibly cytotoxicity. Next, we generated two kinds of *TK1* gene knockout DLD1 cells using the CRISPR/Cas9 system to target exon 1 or exon 4 (Supplementary Fig. S3A and S3B). Several clones with either exon 1 or exon 4 disrupted in both alleles were obtained (data not shown), and the complete loss of TK1 protein expression was confirmed (Fig. 3A). Clones derived from targeting each of the two exons (DLD1-*TK1*$^{-/-}$ #1-1 and DLD1-*TK1*$^{-/-}$ #2-1) were selected and shown to be proliferated normally (Supplementary Fig. S3C and S3D). Then, these knockout DLD1 cell lines were subjected to the FTD sensitivity assay, which showed that the FTD IC_{50} values in DLD1-*TK1*$^{-/-}$ #1-1 and DLD1-*TK1*$^{-/-}$ #2-1 cells were comparable with that in DLD1-FTD cells (Table 2). Consistent with these findings, we hardly detected FTD incorporation in DLD1-*TK1*$^{-/-}$ #1-1 or DLD1-*TK1*$^{-/-}$ #2-1 cells even after exposure to a high concentration of FTD (10 μmol/L; Fig. 3B and C). These results clearly show that complete loss of the TK1 protein confers severe FTD resistance by impeding FTD incorporation into the DNA of proliferating cells, and strongly indicate that there are no redundant factors that complement the function of TK1 in FTD activation and cytotoxicity.

DLD1-FTD and DLD1-*TK1*$^{-/-}$ cells do not exhibit 5-FU resistance

The pyrimidine analogue 5-FU is a classical anticancer drug that exerts cytotoxicity by compromising the pyrimidine biosynthesis pathway (17). It was previously shown that DLD1-FTD cells do not exhibit 5-FU resistance (12). This was confirmed using the present assay system, which showed that the IC_{50} values for 5-FU in DLD1 and DLD1-FTD cells were comparable (Table 2). Furthermore, the IC_{50} values for 5-FU in DLD1-*TK1*$^{-/-}$ #1-1 and DLD1-*TK1*$^{-/-}$ #2-1 cells were comparable with those in DLD1 and DLD1-FTD cells (Table 2). This result indicates that loss of TK1 expression does not affect 5-FU cytotoxicity.

The cytotoxicity of 5-FU is mediated both by its incorporation into RNA and by fluorodeoxyuridine monophosphate (FdUMP)–mediated TS inhibition (17). The incorporation of 5-FU into RNA is responsible for its toxic side effects and the FdUMP-mediated inhibition of TS activity for the antitumor effect (18). 5-FU is activated by conversion to fluorouridine monophosphate (FUMP) catalyzed by orotate phosphoribosyltransferase (OPRT) and its subsequent phosphorylation to produce fluorouridine triphosphate (FUTP), enabling its incorporation into RNA (Fig. 4A). A proportion of fluorouridine diphosphate (FUDP) is converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RNR), and FdUDP is further converted into

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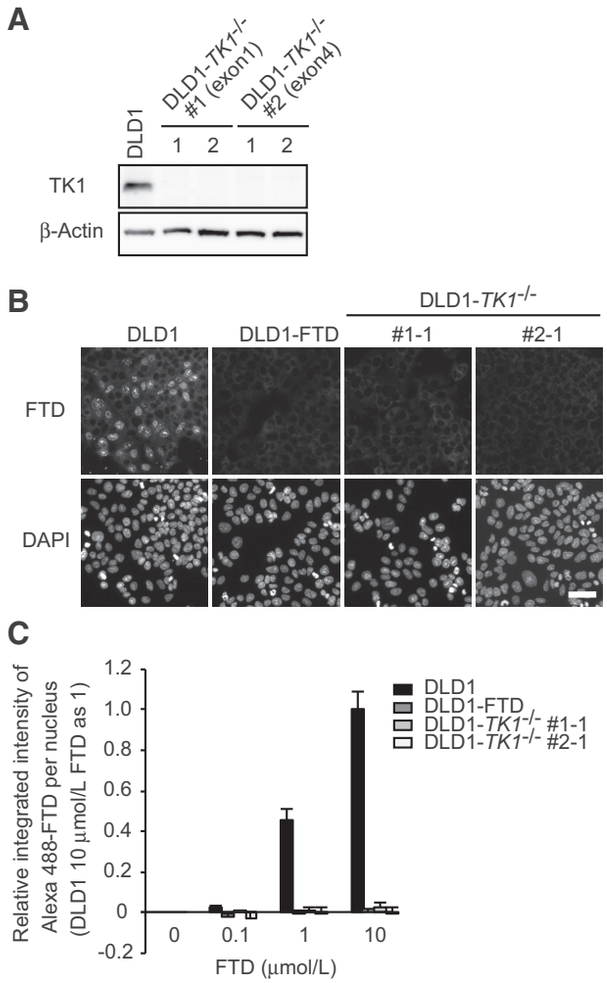

Figure 3.

Knocking out the *TK1* gene confers a defect in FTD metabolism similar to that of DLD1-FTD cells. **A,** TK1 protein expression in DLD1 and CRISPR/Cas9–mediated *TK1* gene knockout cells targeted to exon 1 and exon 4. Immunoblots using antibodies against the indicated proteins are shown. **B,** Fluorescent immunostaining of FTD incorporated into DNA as detected by the anti-BrdUrd antibody, 3D4. Nuclei were stained with DAPI. Representative images of each cell line exposed to 10 μmol/L FTD for 1 hour are shown. Scale bar, 50 μm. **C,** Relative integrated fluorescence of the FTD signal per nucleus. Bar graphs indicate the average of the relative scores of the integrated fluorescence intensity in each individual cell when the average score of DLD1 cells cultured in the presence of 10 μmol/L FTD for 1 hour is defined as 1. Error bars represent the SD of three independent experiments.

fluorodeoxyuridine triphosphate (FdUTP) and FdUMP, which inhibits TS activity via ternary complex formation with TS and 5,10-methylene tetrahydrofolate (mTHF; Fig. 4A; ref. 17). Another possible pathway of 5-FU activation is the sequential action of TP and TK (Fig. 4A); however, the contribution of this pathway should be minimal in DLD1 and DLD1-FTD cells because the TP protein (Supplementary Fig. S1B) or *TYMP* mRNA (Supplementary Fig. S1C) was hardly detected in these cells. To examine whether the 5-FU metabolic pathway was intact in the FTD-resistant cells, the kinetics of FdUMP–TS–mTHF ternary complex formation were measured in 5-FU–treated DLD1, DLD1-FTD, and DLD1-*TK1*$^{-/-}$ cells. There was no significant difference between the cell lines (Fig. 4B), suggesting that DLD1-FTD and DLD1-*TK1*$^{-/-}$ cells can activate 5-FU properly. Next, the intracellular metabolites of the pyrimidine biosynthesis pathway were quantified by LC-QqQ-MS (Fig. 4C). No differences in the amount of pyrimidine ribonucleotides including UMP, UDP, UTP, and CTP were observed in DLD1-FTD or DLD1-*TK1*$^{-/-}$ cells (Fig. 4D). In addition, the FdUMP–TS–mTHF ternary complex was stably detected for 48 hours both in DLD1 and DLD1-FTD cells once it was formed (Supplementary Fig. S4). These data indicate that FTD resistance or the loss of TK1 protein expression does not alter the cellular metabolism that would affect 5-FU activation or cytotoxicity.

Table 2. IC_{50} concentration of FTD, 5-FU, and FdUrd in DLD1 and *TK1* knockout cells

Cell lines	FTD (μmol/L)	5-FU (μmol/L)	FdUrd (μmol/L)
DLD1	7.8 ± 1.60	3.80 ± 0.45	0.15 ± 0.05
DLD1-FTD	>300	3.70 ± 0.43	>30
DLD1 *TK1*$^{-/-}$ #1-1	>300	5.00 ± 1.13	>30
DLD1 *TK1*$^{-/-}$ #2-1	>300	4.90 ± 1.33	>30

DLD1-FTD and DLD1-*TK1*$^{-/-}$ cells exhibit FdUrd resistance

FdUrd is a deoxyribonucleoside form of 5-FU, and previous reports show that DLD1-FTD cells exhibit FdUrd resistance (12). To test whether TK1 is involved in the resistance to FdUrd, DLD1 and DLD1-FTD cells were exposed to FdUrd, and the IC_{50} was determined. DLD1-FTD cells showed severe FdUrd resistance (Table 2). DLD1-*TK1*$^{-/-}$ #1-1 and DLD1-*TK1*$^{-/-}$ #2-1 cells showed comparable levels of FdUrd resistance (Table 2). These results indicate that loss of TK1 expression confers severe FdUrd resistance.

The cytotoxicity of FdUrd is attributed to FdUMP-mediated TS inhibition and FdUTP incorporation into DNA (Fig. 5A). FdUrd is directly converted to FdUMP by phosphorylation, which is mediated by the action of nucleoside kinases including TK1 (19). To test whether TK1 is involved in the process of FdUMP biogenesis from FdUrd, DLD1, DLD1-FTD, and DLD1-*TK1*$^{-/-}$ cells were treated with 0.2 μmol/L FdUrd and the kinetics of FdUMP–TS–mTHF ternary complex formation was monitored. In DLD1 cells, the ternary complex was fully formed at 4 hours and the level did not change after 12 hours (Fig. 5B). In contrast, in DLD1-FTD and DLD1 *TK1*$^{-/-}$ #1-1 cells, the ternary complex was formed but at a low level throughout (Fig. 5B). Because TS-targeted agents are used to treat numerous solid and hematologic malignancies (20), the inhibition of TS activity is believed to be a cause of cytotoxicity. To test whether the difference in the efficiency of FdUMP–TS–mTHF ternary complex formation among these cell lines is correlated with FdUrd-induced cell death, the sub-G_1 cell population was measured in FdUrd-treated DLD1 cells and their derivatives. FdUrd increased the sub-G_1 cell population in the parental DLD1 cells, but not in DLD1-FTD or DLD1-*TK1*$^{-/-}$ cells (Fig. 5C). In contrast, no increase in the sub-G_1 cell population was observed in 5-FU–treated DLD1 cells or their derivatives (Fig. 5C), suggesting that the antiproliferating effect of 5-FU on these cells is not mediated by apoptosis. Collectively, these results indicate that TK1 is critically involved in the process of FdUMP production from FdUrd and contributes to FdUrd-induced cell death and cytotoxicity.

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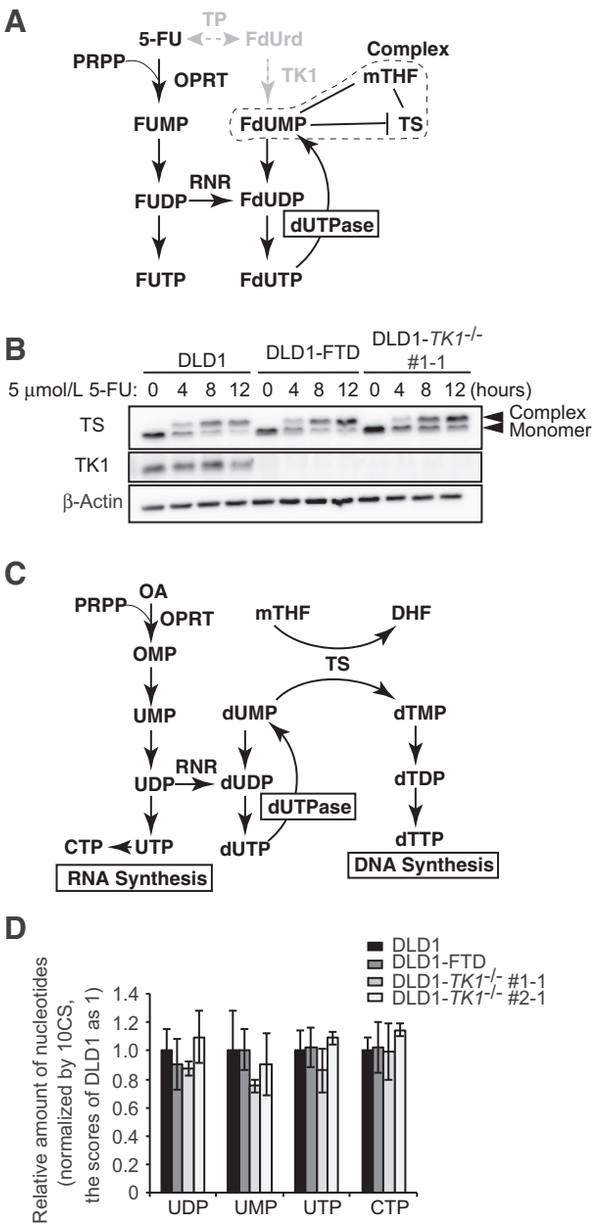

Figure 4.

FTD-resistant cells do not exhibit the defect in 5-FU metabolism. **A,** Schematic representation of 5-FU metabolism. Letters and lines shown in gray color indicate the factors and pathways, respectively, considered of little contribution. The ternary complex of FdUMP, TS, and mTHF is surrounded by the dotted line. **B,** Kinetics of FdUMP–TS–mTHF ternary complex (complex) formation in 5-FU–treated DLD1, DLD1-FTD, and DLD1-*TK1*$^{-/-}$ cells. Monomer indicates the TS monomer. Immunoblots using antibodies against the indicated proteins are shown. **C,** Schematic view of the pyrimidine biosynthesis pathway. **D,** Relative quantity of pyrimidine nucleotides in DLD1-FTD and DLD1-*TK1*$^{-/-}$ cells. Bar graphs indicate the average of the quantity of pyrimidine nucleotides relative to that in DLD1 cells (considered as a value of 1). Error bars represent the SD of four independent experiments. No statistically significant difference was found in any combination. 10CS, L(+)-10-camphor sulfonic acid; DHF, dihydrofolate; OA, orotic acid; OMP, oritidine monophosphate; PRPP, phosphoribosyl pyrophosphate.

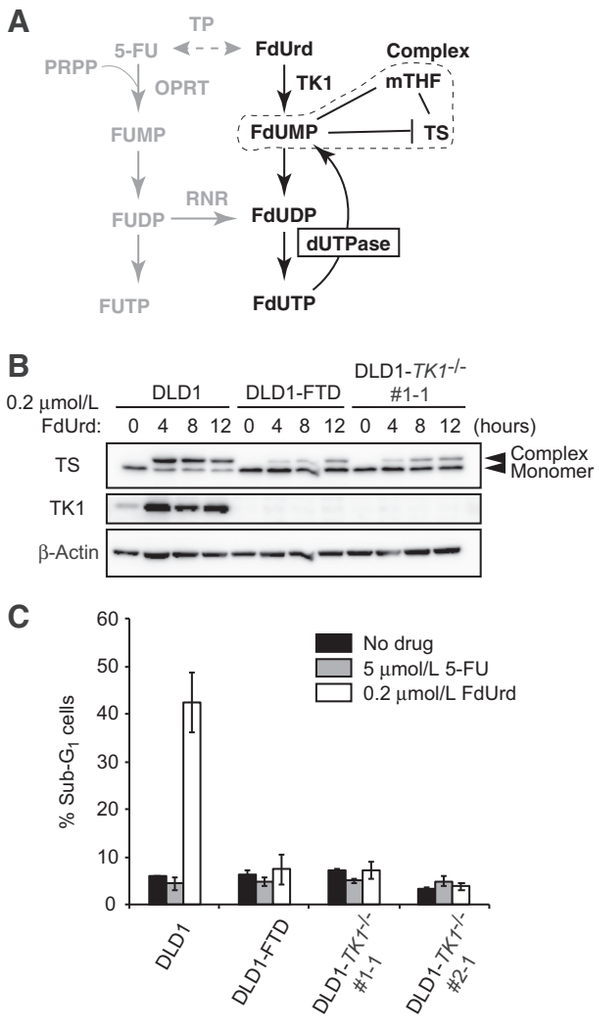

Figure 5.

DLD1-FTD and DLD1-*TK1*$^{-/-}$ cells are deficient in FdUrd metabolism. **A,** Schematic representation of FdUrd metabolism. Letters and lines shown in gray color indicate the factors and pathways, respectively, considered of little contribution. The ternary complex of FdUMP–TS–mTHF is surrounded by the dotted line. **B,** Kinetics of FdUMP–TS–mTHF ternary complex (complex) formation in FdUrd-treated DLD1, DLD1-FTD, and DLD1-*TK1*$^{-/-}$ cells. Monomer indicates the TS monomer. Immunoblots using antibodies against the indicated proteins are shown. **C,** Apoptotic cell death induced by FdUrd, but not by 5-FU. The proportion of the sub-G_1 cell population was measured at 48 hours.

Discussion

Acquired resistance to therapeutic drugs is a devastating problem for patients receiving long-term systemic treatment. In this study, we investigated FTD resistance *in vitro* in DLD1 cells rendered FTD resistant by repeated, continuous exposure in culture to escalating concentrations of the drug. In DLD1-FTD cells, we identified a nonsense mutation within the coding exon of the *TK1* gene resulting in the production of a truncated and possibly nonfunctional protein. In addition, specific and complete loss of the functional TK1 protein by gene knockout using the CRISPR/Cas9 system conferred severe FTD resistance. These

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data demonstrate that TK1 is a critical and nonredundant cellular component that determines FTD cytotoxicity. Furthermore, similar to DLD1-FTD cells, DLD1-*TK1*$^{-/-}$ cells did not exhibit any cross-resistance to 5-FU, which implies that 5-FU–based therapy should be clinically effective in patients refractory to TFTD.

Why does loss of TK1 confer FTD resistance? Similar to thymidine, once FTD is transported into the cytoplasm of tumor cells by nucleoside transporters, hENT1, hENT2 (5, 6), or hCNT1 (21), it is activated by sequential phosphorylation by TK, TYMK, and NDK. FTD cytotoxicity is possibly mediated by the inhibition of TS activity by FTD-MP (10) and the incorporation of FTD-TP into DNA (7–9). Our data clearly showed that TK1, a nuclear isoform of TK, plays a crucial role in FTD activation in the nucleus (Figs. 2 and 3). Loss of the functional TK1 protein led to the development of FTD resistance (Fig. 3), which may be caused by the inhibition of the initial phosphorylation step and the production of cytotoxic FTD-MP and FTD-TP in the nucleus. In addition to the loss or decreased expression of the TK1 protein, the downregulation of nucleoside transporters and upregulation of sPLA2 are associated with FTD resistance (4). These results suggest that the impairment of either step in the FTD activation pathway would cause FTD resistance. In the clinical setting, the deficiency of TK1 or nucleoside transporters in tumors might cause resistance to TFTD in patients because of the lack of FTD activation. In support of this notion, sequence analysis of genomic DNA from peripheral whole blood of the patients who received TFTD medication suggested the potential roles of SNPs of nucleoside transporter genes in predicting TFTD efficacy and toxicity (22).

The *TK1* gene mutation we identified in DLD1-FTD cells (c.740G>T(G177*)) was a G-to-T transversion (Fig. 1A and B). We presume that this mutation was introduced accidentally and that the cells that gained this mutation and lost the wild-type allele survived the escalating concentrations of FTD in culture. Theoretically, the G-to-T transversion occurs when A is misincorporated at the position of an oxidized form of guanine (8-oxoG) in the template strand (23). FTD is incorporated into DNA at the A position in the template strand in the first S-phase. In the next S-phase, G is often misincorporated at the position of FTD in the template strand. This would result in an A-to-G transition rather than a G-to-T transversion. Because the frequency of spontaneous mutations is high in tumors with mismatch repair deficiency, the likelihood of acquiring FTD resistance may be higher in tumors with high microsatellite instability (MSI-H) type than in microsatellite stable tumors. Intriguingly, the only FTD-resistant human colorectal cancer cell lines established to date are of MSI-H type (12, 14).

We confirmed that neither DLD1-FTD cells or DLD1-*TK1*$^{-/-}$ cells exhibited any cross-resistance to 5-FU (Table 2). Similar results were reported in previous studies (4, 11), suggesting that severe FTD resistance does not cause 5-FU cross-resistance. In addition to TK1 loss, decreased hENT and increased sPLA2 were the causes of FTD resistance but not of 5-FU resistance (4); this indicates that hENT is not required for the transport of 5-FU into the cytoplasm, and phospholipid metabolism is not involved in 5-FU metabolism or cytotoxicity. Within the cytoplasm of cells, 5-FU is mainly activated by OPRT and converted to the ribonucleotide form, FUMP. FUMP is further phosphorylated to FUDP and FUTP, and a proportion of FUDP is converted to the deoxyribonucleotide form, FdUDP, by RNR (Fig. 4A). The sequential action of TP and TK1 should contribute minimally in 5-FU activation, at least in DLD1 cells and their derivatives, because TP expression was hardly detected in these cells (Supplementary Fig. S1B and S1C). In our analysis of DLD1-FTD cells and DLD1-*TK1*$^{-/-}$ cells, the amount of intracellular pyrimidine ribonucleotides (UMP, UDP, UTP, and CTP) was comparable with that of the parent DLD1 cells (Fig. 4D), and the kinetics of FdUMP–TS–mTHF ternary complex formation were not altered (Fig. 4B). These data indicate that chronic exposure to FTD or acquired resistance to FTD does not affect the 5-FU metabolic pathway. However, further analysis using other cell lines with acquired resistance to FTD will be necessary to verify this concept in TP-expressing cells, because DLD1 cells may lack TP protein expression (Supplementary Fig. S1B and S1C).

The current results confirmed that both DLD1-FTD cells and DLD1-*TK1*$^{-/-}$ cells exhibited severe FdUrd resistance (Table 2). The fact that cross-resistance to FdUrd in FTD-resistant cells is present in other cell lines (4) suggests that FTD and FdUrd share a common activation mechanism, namely hENT-mediated transport and TK1-mediated activation. The formation of the FdUMP–TS–mTHF ternary complex was saturated after a short (4 hours) exposure to 0.2 μmol/L FdUrd (Fig. 5B), suggesting that FdUrd is quickly and efficiently converted to FdUMP by TK1-mediated phosphorylation (Fig. 5A). In the absence of TK1, FdUMP–TS–mTHF ternary complex formation was significantly suppressed, but still observed, albeit inefficiently and with slow kinetics (Fig. 5B). This residual FdUMP–TS–mTHF ternary complex formation might be achieved either by TP-mediated FdUrd conversion to 5-FU, which can be activated via successive conversion to FUMP, FUDP, FdUDP, FdUTP, and FdUMP (Fig. 5A), or through the action of a different kinase (such as TK2) catalyzing FdUrd conversion to FdUMP. The inefficiency of FdUMP–TS–mTHF ternary complex formation by FdUrd in DLD1-FTD and DLD1-*TK1*$^{-/-}$ cells could be caused by the absence of TP in these cells. Similarly, although FdUrd is the deoxyribonucleoside form of 5-FU, the amount of 5-FU converted directly to FdUrd should be small in DLD1 cells and their derivatives (Fig. 4A), because TP activity would be necessary for the reaction. These results indicate that, at least in DLD1 cells and their derivatives, the majority of 5-FU and FdUrd are mostly not interconvertible and support the idea that 5-FU and FdUrd have different mechanisms of action (24). It is important to verify that this concept is also applicable to TP-expressing tumor cells.

In conclusion, our genetic analysis identified TK1 as a unique and nonredundant cellular component that determines FTD cytotoxicity. It is encouraging that 5-FU maintains its toxicity in cells with acquired resistance to FTD. Current TFTD regimens are approved only for patients with metastatic colorectal cancer who are refractory to 5-FU or other standard drugs (2). Clinical trials for regimens including TFTD as first-line treatment for patients with metastatic colorectal cancer are currently underway (UMIN000025241). When TFTD medication is given to patients who have not received any prior therapy, we can expect that 5-FU–based regimens will remain effective in patients who become refractory to TFTD. Future clinical trials may verify this concept and facilitate the development of novel chemotherapeutic regimens.

Disclosure of Potential Conflicts of Interest

E. Oki has received speakers bureau honoraria from Taiho Pharmaceutical Co. Ltd. H. Kitao reports receiving a commercial research grant from Taiho Pharmaceutical Co. Ltd. Y. Maehara reports receiving a commercial research

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Writing, review, and/or revision of the manuscript: K. Edahiro, T. Kobunai, E. Oki, H. Kitao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Iimori, D. Miura, Y. Kataoka
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References

1. Yoshino T, Mizunuma N, Yamazaki K, Nishina T, Komatsu Y, Baba H, et al. TAS-102 monotherapy for pretreated metastatic colorectal cancer: a double-blind, randomised, placebo-controlled phase 2 trial. Lancet Oncol 2012;13:993–1001.
2. Mayer RJ, Van Cutsem E, Falcone A, Yoshino T, Garcia-Carbonero R, Mizunuma N, et al. Randomized trial of TAS-102 for refractory metastatic colorectal cancer. N Engl J Med 2015;372:1909–19.
3. Emura T, Suzuki N, Fujioka A, Ohshimo H, Fukushima M. Potentiation of the antitumor activity of alpha, alpha, alpha-trifluorothymidine by the co-administration of an inhibitor of thymidine phosphorylase at a suitable molar ratio in vivo. Int J Oncol 2005;27:449–55.
4. Temmink OH, Bijnsdorp IV, Prins HJ, Losekoot N, Adema AD, Smid K, et al. Trifluorothymidine resistance is associated with decreased thymidine kinase and equilibrative nucleoside transporter expression or increased secretory phospholipase A2. Mol Cancer Ther 2010;9:1047–57.
5. Sakamoto K, Yokogawa T, Ueno H, Oguchi K, Kazuno H, Ishida K, et al. Crucial roles of thymidine kinase 1 and deoxyUTPase in incorporating the antineoplastic nucleosides trifluridine and 2'-deoxy-5-fluorouridine into DNA. Int J Oncol 2015;46:2327–34.
6. Kitao H, Morodomi Y, Niimi S, Kiniwa M, Shigeno K, Matsuoka K, et al. The antibodies against 5-bromo-2'-deoxyuridine specifically recognize trifluridine incorporated into DNA. Sci Rep 2016;6:25286.
7. Emura T, Nakagawa F, Fujioka A, Ohshimo H, Yokogawa T, Okabe H, et al. An optimal dosing schedule for a novel combination antimetabolite, TAS-102, based on its intracellular metabolism and its incorporation into DNA. Int J Mol Med 2004;13:249–55.
8. Matsuoka K, Iimori M, Niimi S, Tsukihara H, Watanabe S, Kiyonari S, et al. Trifluridine induces p53-dependent sustained G2 phase arrest with its massive misincorporation into DNA and few DNA strand breaks. Mol Cancer Ther 2015;14:1004–13.
9. Tanaka N, Sakamoto K, Okabe H, Fujioka A, Yamamura K, Nakagawa F, et al. Repeated oral dosing of TAS-102 confers high trifluridine incorporation into DNA and sustained antitumor activity in mouse models. Oncol Rep 2014;32:2319–26.
10. Eckstein JW, Foster PG, Finer-Moore J, Wataya Y, Santi DV. Mechanism-based inhibition of thymidylate synthase by 5-(trifluoromethyl)-2'-deoxyuridine 5'-monophosphate. Biochemistry 1994;33:15086–94.
11. Temmink OH, Comijn EM, Fukushima M, Peters GJ. Intracellular thymidylate synthase inhibition by trifluorothymidine in FM3A cells. Nucleosides Nucleotides Nucleic Acids 2004;23:1491–4.
12. Murakami Y, Kazuno H, Emura T, Tsujimoto H, Suzuki N, Fukushima M. Different mechanisms of acquired resistance to fluorinated pyrimidines in human colorectal cancer cells. Int J Oncol 2000;17:277–83.
13. Emura T, Murakami Y, Nakagawa F, Fukushima M, Kitazato K. A novel antimetabolite, TAS-102 retains its effect on FU-related resistant cancer cells. Int J Mol Med 2004;13:545–9.
14. Tsunekuni K, Konno M, Asai A, Koseki J, Kobunai T, Takechi T, et al. MicroRNA profiles involved in trifluridine resistance. Oncotarget 2017;8:53017–27.
15. Kiyonari S, Iimori M, Matsuoka K, Watanabe S, Morikawa-Ichinose T, Miura D, et al. The 1,2-Diaminocyclohexane carrier ligand in oxaliplatin induces p53-dependent transcriptional repression of factors involved in thymidylate biosynthesis. Mol Cancer Ther 2015;14:2332–42.
16. Emura T, Suzuki N, Yamaguchi M, Ohshimo H, Fukushima M. A novel combination antimetabolite, TAS-102, exhibits antitumor activity in FU-resistant human cancer cells through a mechanism involving FTD incorporation in DNA. Int J Oncol 2004;25:571–8.
17. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 2003;3:330–8.
18. Pinedo HM, Peters GF. Fluorouracil - biochemistry and pharmacology. J Clin Oncol 1988;6:1653–64.
19. Pettersen HS, Visnes T, Vagbo CB, Svaasand EK, Doseth B, Slupphaug G, et al. UNG-initiated base excision repair is the major repair route for 5-fluorouracil in DNA, but 5-fluorouracil cytotoxicity depends mainly on RNA incorporation. Nucleic Acids Res 2011;39:8430–44.
20. Wilson PM, Danenberg PV, Johnston PG, Lenz HJ, Ladner RD. Standing the test of time: targeting thymidylate biosynthesis in cancer therapy. Nat Rev Clin Oncol 2014;11:282–98.
21. Okayama T, Yoshisue K, Kuwata K, Komuro M, Ohta S, Nagayama S. Involvement of concentrative nucleoside transporter 1 in intestinal absorption of trifluorothymidine, a novel antitumor nucleoside, in rats. J Pharmacol Exp Ther 2012;340:457–62.
22. Suenaga M, Schirripa M, Cao S, Zhang W, Yang D, Dadduzio V, et al. Potential role of polymorphisms in the transporter genes ENT1 and MATE1/OCT2 in predicting TAS-102 efficacy and toxicity in patients with refractory metastatic colorectal cancer. Eur J Cancer 2017;86:197–206.
23. Ohno M, Sakumi K, Fukumura R, Furuichi M, Iwasaki Y, Hokama M, et al. 8-oxoguanine causes spontaneous *de novo* germline mutations in mice. Sci Rep 2014;4:4689.
24. van Laar JA, Rustum YM, Ackland SP, van Groeningen CJ, Peters GJ. Comparison of 5-fluoro-2'-deoxyuridine with 5-fluorouracil and their role in the treatment of colorectal cancer. Eur J Cancer 1998;34:296–306.

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