dUTPase inhibition confers susceptibility to a thymidylate synthase inhibitor in DNA-repair-defective human cancer cells

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Abstract
Deficiency in DNA repair proteins confers susceptibility to DNA damage, making cancer cells vulnerable to various cancer chemotherapies. 5-Fluorouracil (5-FU) is an anticancer nucleoside analog that both inhibits thymidylate synthase (TS) and causes DNA damage via the misincorporation of FdUTP and dUTP into DNA under the conditions of dTTP depletion. However, the role of the DNA damage response to its antitumor activity is still unclear. To determine which DNA repair pathway contributes to DNA damage caused by 5-FU and uracil misincorporation, we examined cancer cells treated with 2'-deoxy-5-fluorouridine (FdUrd) in the presence of TAS-114, a highly potent inhibitor of dUTPase that restricts aberrant base misincorporation. Addition of TAS-114 increased FdUTP and dUTP levels in HeLa cells and facilitated 5-FU and uracil misincorporation into DNA, but did not alter TS inhibition or 5-FU incorporation into RNA. TAS-114 showed synergistic potentiation of FdUrd cytotoxicity and caused aberrant base misincorporation, leading to DNA damage and induced cell death even after short-term exposure to FdUrd. Base excision repair (BER) and homologous recombination (HR) were found to be involved in the DNA repair of 5-FU and uracil misincorporation caused by dUTPase inhibition in genetically modified chicken DT40 cell lines and siRNA-treated HeLa cells. These results suggested that BER and HR are major pathways that protect cells from the antitumor effects of massive incorporation of 5-FU and uracil. Further, dUTPase inhibition has the potential to maximize the antitumor activity of fluoropyrimidines in cancers that are defective in BER or HR.

KEYWORDS
5-fluorouracil/uracil misincorporation, base excision repair, DNA repair, dUTPase, homologous recombination
1 | INTRODUCTION

Genome integrity is preserved by sophisticated mechanisms coordinated by a highly conserved and well organized system of proteins that prevents and repairs DNA damage. Accumulation of gene abnormalities is a hallmark of cancer cells and leads to phenotypes such as continuous proliferation, metastasis, and resistance to chemotherapy. An underlying mechanism of carcinogenesis is abnormality or deficiency in DNA repair, as evidenced by DNA mismatch repair (MMR) deficiency in colorectal cancer and homologous recombination repair (HR) deficiency because of breast cancer susceptibility gene (BRCA1/2) mutations in breast and ovarian cancers.

Although the accumulation of gene abnormalities causes carcinogenesis and cancer progression, DNA repair deficiency sometimes makes the cancer vulnerable to various cancer therapies. For instance, poly(ADP-ribose) polymerase (PARP) inhibitors can be used to target cancer cells that lack appropriate DNA double-stranded repair mechanisms due to a deficiency in proteins essential for HR, eg, BRCA1 and BRCA2.

Although DNA repair deficiency provides opportunities for cancer therapy, overexpression of DNA repair proteins causes resistance to chemotherapies. For example, excessive expression of excision repair cross-complementation group 1 (ERCC1), a key player in the nucleotide excision repair (NER) pathway, appears to be responsible for cisplatin resistance in non-small-cell lung cancer. O6-Methylguanine-DNA methyltransferase plays a crucial role in the removal of modified bases in DNA necessary for the antitumor activity of alkylating agents.

In addition to DNA repair proteins, enzymes that prevent DNA damage also contribute to chemotherapy resistance. Deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase) is a catabolic enzyme in the pyrimidine metabolic pathway that degrades dUTP to dUMP and prevents innate uracil misincorporation into DNA. Although uracil is not a component of DNA, DNA polymerase can utilize dTTP and dUTP with equal efficiency in DNA synthesis. Therefore, dUTPase maintains the intracellular dUTP pool at an extremely low level to minimize uracil misincorporation into DNA.

5-Fluorouracil (5-FU), which is a cornerstone of colorectal cancer therapy, has multiple modes of action because of its active metabolites. Thymidylate synthase (TS) is the rate-limiting enzyme in the conversion of dUMP to dTMP and is responsible for de novo dTTP synthesis. The primary mode of action of 5-FU is believed to be the inhibition of TS, which is mediated by the formation of a ternary complex of 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) with TS and 5,10-methylenetetrahydrofolate, leading to dTTP depletion. When TS is inhibited, massive amounts of its substrate, dUMP, accumulate and become phosphorylated to form dUTP. In addition, 5-FU undergoes conversion to 5-fluoro-2'-deoxyuridine 5'-triphosphate (FdUTP), which, like dUTP, can be misincorporated into DNA. Although misincorporation of FdUTP and dUTP into DNA is considered to be one mechanism involved in 5-FU-based chemotherapy, these two nucleotides are undetectable or are present at very low concentrations in cancer cells under physiological conditions because they are dUTPase substrates and are immediately converted to their respective monophosphates that cannot be misincorporated into DNA. An elevated expression of dUTPase is observed in various cancers, and it has been suggested that higher expression results in resistance to 5-FU chemotherapy because overproduction of dUTPase limits the misincorporation of FdUTP and dUTP into DNA, hence, dUTPase is a potential target for improving this chemotherapy's efficacy.

We had previously demonstrated that TAS-114, a small-molecule inhibitor of dUTPase, can significantly enhance the antitumor activity of 5-FU in various preclinical models. TAS-114 can specifically modulate aberrant base incorporation into DNA in cancer cells when TS is inhibited by 5-FU, and inhibition of dUTPase plays a crucial role in the enhancement of 5-FU-mediated antiproliferative activity (Figure 1).

Studies have demonstrated the relationship between DNA repair proteins and misincorporation of aberrant bases caused by 5-FU treatment in cancer cells. However, these studies were conducted under conditions of low concentrations of FdUTP and dUTP that are maintained at low levels by dUTPase and are substrates of DNA polymerases.

In this study, we aimed to analyze the DNA damage response in cancer cells after an increase in aberrant base misincorporation under the conditions of dUTPase inhibition. In particular, we explored the DNA repair pathways whose deficiency/inhibition can be crucial for the antitumor-enhancing activity mediated by dUTPase inhibition.

2 | MATERIALS AND METHODS

2.1 | Chemical compounds

TAS-114 [N-[(1R)-1-[3-(cyclopentyloxy)phenyl]-ethyl]-3-[(3,4-dihydro-2,4-dioxo-1(2H)-pyrimidinyl)methoxy]-1-propanesulfonamide] (see ref. (20) for chemical structure and method of synthesis) was synthesized at Taiho Pharmaceutical Co., Ltd.; 5-FU and paclitaxel were obtained from Wako Pure Chemical Industries, Ltd. 2'-deoxy-5-fluorouridine (FdUrd) was obtained from Wako Pure Chemical Industries, Ltd. and Carbonsynth Limited. [6-3H]-FdUMP (666 GBq/mmol), [3H]-FdUrd (614 GBq/mmol), and [3H]-5-FU (570 GBq/mmol) were obtained from Moravek Biochemicals, Inc.

2.2 | Cell lines

The human cervical cancer-derived HeLa cell line was obtained from the Health Science Research Resources Bank and re-authenticated in 2012 by short tandem repeat-based DNA profiling. The chicken DT40 cell lines (Supporting Information Table S1) used in this study
were kindly provided by Shunichi Takeda (Kyoto University) and Patricia Gearhart (National Institute on Aging).

2.3 | Measurement of intracellular nucleotide pool

HeLa cells (5 x 10^6 cells) were seeded into 175-cm² flasks and cultured for 24 h. Compounds were added, and the cells were incubated for 4 or 8 h and collected as cell pellets. The cell pellets were suspended in 200 μL of 0.48 N perchloric acid by vortexing, the suspension was centrifuged, and 600 μL of a dichloromethane solution containing 0.5 N tri-n-octylamine was added to the supernatant and mixed by vortexing. Following centrifugation, the aqueous layer was collected and analyzed as the acid-soluble fraction. The amounts of dUMP, dTTP, and NAD⁺ in the acid-soluble fraction were quantified using a Shimadzu LC-VP Series HPLC system equipped with a UV detector set to 254 nm. Amounts of FdUTP and 5'-fluorouridine 5'-triphosphate (FUTP) were quantified using a radio-HPLC system (Flow Scintillation Analyzer 525TR; Packard Bioscience), as described in our previous report. The dUTP levels were measured using a PCR thermocycler-based fluorescence-based assay developed by Wilson et al.

2.4 | Measurement of intracellular free-TS levels

As described earlier, HeLa cells were cultured and incubated with compounds for the measurement of the intracellular nucleotide pool. Cell pellets were homogenized, centrifuged at 105 000 g for 1 h at 4°C, and the supernatants were collected. Intracellular free-TS levels (pmol/mg protein) were measured using a modified FdUMP-TS binding assay developed by Takeda et al.; see our previous report.

2.5 | Measurement of 5-FU incorporation into DNA and RNA

HeLa cells were seeded into 75-cm² flasks (2 x 10^6 cells) and in 6-well plates (2.5 x 10^5 cells/well) for quantification of 5-FU incorporated into DNA and RNA, respectively. Compounds were added at 24 h after cell seeding, and the cells were incubated for 8 h and collected as cell pellets. DNA was extracted from the cell pellets using a DNeasy Blood and Tissue kit (QIAGEN). RNA was extracted from the cells using an RNeasy Plus Mini kit (QIAGEN). The concentrations of DNA or RNA in the solution were estimated from the absorbance at 260 nm. Radioactivity was measured using a Liquid Scintillation Analyzer Tri-Carb 2900TR (Perkin Elmer), and the amounts of [³H]-5-FU per μg of DNA or RNA were calculated.

2.6 | Measurement of cell viability

HeLa cells were seeded into 12-well plates (5 x 10^4 cells/well) and cultured for 24 h. Compounds were added and the viable and dead cells were counted prior to and at 8, 12, 24, 36, or 48 h after compound addition using the trypan blue exclusion method.

2.7 | Western blotting

HeLa cells were seeded into 6-well plates (2 x 10^5 cells/well) and cultured for 24 h. Compounds were added, and the cells were incubated...
for 8, 16, or 24 h. Cells were lysed in lysis buffer (M-PER Mammalian Protein Extraction Reagent [Thermo Fisher Scientific Inc] supplemented with Complete, Mini, Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail [Roche Diagnostics K. K.]). Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc). Membranes were blocked with Blocking One reagent (Nacalai Tesque, Inc) and probed with the appropriate primary antibodies. The following primary antibodies were purchased from Cell Signaling Technology, Inc: anti-cleaved caspase-3 (catalog no. 9661; dilution, 1:1000); anti-cleaved caspase-9 (9501, 1:1000); anti-cleaved PARP (5625, 1:1000); and anti-β-actin (4967, 1:2000). Anti-TS (catalog no. 10409; dilution, 1:4000) was purchased from Immuno-Biological Laboratories Co, Ltd. The membranes were then incubated with horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology, Inc), and proteins were visualized by luminol-based enhanced chemiluminescence. Luminescence images were captured with a LAS 4010 imaging system (GE Healthcare UK Ltd.).

2.8 | Evaluation of relative cellular sensitivity using a DT40 mutant cell panel

Cells of the DT40 mutant panel (Table S1) were plated into 96-well plates and cultured for 24 h. Compounds were added, and the cells were incubated for 72 h. Cell viability was evaluated using the CellTiter-Glo assay (Promega Corp.). IC50 values (concentration at which 50% inhibition of cell viability was observed relative to untreated control) were calculated, and the sensitivity in each mutant cell line (sensitivity score) was determined relative to the wild-type (WT) cell line. The exception was the AID−/− UNG−/− cell line (deficient in activation-induced cytidine deaminase (AID) and uracil-DNA glycosylase (UNG)), for which the sensitivity was determined relative to the AID−/− cell line. The sensitivity score was converted to a logarithmic scale (base 2) using the following formula: Sensitivity score = log2(Y/X), where Y = IC50 of mutant cells and X = IC50 of WT cells (or AID−/− cells, if applicable).

2.9 | Evaluation of relative sensitivity by siRNA analysis in HeLa cells

Negative control siRNA (Negative control #1) and siRNAs against the following genes were purchased from Thermo Fisher Scientific Inc: UNG (catalog no. s14678), BRCA1 (s459), BRCA2 (s2085), MutL Homology 1 (MLH1, s224047), and ERCC Excision Repair 1 (ERCC1, s4785). siRNA against DNA polymerase β (POLB, catalog no. D005164-01-0002) was purchased from Dharmacon. HeLa cells (1 × 10^3 cells) were seeded into 75-cm² flasks and cultured for 24 h. The cells were then transfected with 16.7 nmol/L siRNA by using Lipofectamine RNAiMAX Reagent (Life Technologies, Inc) and cultured for 24 h. The transfected cells were then seeded at 2 × 10^3 cells/well into 96-well plates and cultured for 24 h. Compounds were added, and the cells were incubated for 24 h. Thymidine solution was added (final concentration, 30 μmol/L), and the cells were incubated for 48 h. Only BRCA2 siRNA-transfected cells were incubated for 72 h following thymidine addition. Cell viability was evaluated by crystal violet staining, as described previously. IC50 values for cell proliferation were calculated, and the sensitivity in each knockdown cell line was determined relative to negative control cells. The sensitivity score was converted to the logarithmic scale (base 2) using the following formula: Sensitivity score = log2(Y/X), where Y = IC50 of knockdown cells and X = IC50 of negative control cells.

2.10 | Statistical analysis

Dunnett test was used to compare dTTP or NAD⁺ levels between the cells treated with TAS-114-plus-FdUrd and the cells treated with TAS-114 alone, in HeLa cells. Student t test was used to compare the levels of dUMP, dUTP, FUTP, and 5-FU in RNA between the cells treated with TAS-114-plus-FdUrd and the cells treated with FdUrd alone. A P-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | In combination with FdUrd, TAS-114 inhibited dUTPase in HeLa cells and facilitated aberrant base misincorporation into DNA

To examine selective modulation of aberrant base misincorporation into DNA through dUTPase inhibition by TAS-114, we first analyzed TS inhibition and pyrimidine nucleotide pools in HeLa cells treated with TAS-114 in combination with FdUrd (Figure 2A).

A covalent ternary complex formed between TS, FdUMP, and 5,10-methylene-tetrahydrofolate is responsible for FdUMP-mediated inhibition of TS. Treatment with FdUrd alone decreased free-TS; increased intracellular dUMP (a substrate of TS), and decreased dTTP (a metabolite produced by TS activity) in a concentration-dependent manner. FdUrd (1 μmol/L) alone also increased intracellular dUTP; however, FdUTP was undetectable.

In combination with FdUrd, TAS-114 increased the levels of dUTP and FdUTP (both substrates of dUTPase) and dramatically decreased the level of dUMP (a product of dUTPase) compared with treatment with FdUrd alone. In contrast, TAS-114 co-treatment had minimal effects on free-TS and dTPP compared to those with FdUrd alone.

Fluoropyrimidines are metabolized not only to FdUMP, but also to ribonucleotides, which cause RNA-based cytotoxicity. The intracellular levels of the ribonucleotide FUTP, a substrate of RNA polymerase, were not affected by the presence of TAS-114 (Figure 2B).

As expected from the FdUrd-induced dTTP depletion and TAS-114-induced increase in intracellular dUTP and FdUTP (Figure 2A), treatment with FdUrd-plus-TAS-114 increased 5-FU misincorporation into DNA, without affecting its incorporation into RNA when compared with treatment with FdUrd alone (Figure 2C). These results confirmed that TAS-114 inhibited dUTPase in HeLa cells and facilitated aberrant base misincorporation into DNA.
Treating the cells with 5-FU instead of FdUrd yielded similar results, except that the 5-FU-derived ribonucleotide levels were higher compared with those of deoxyribonucleotides due to differences in the metabolic pathways of these fluoropyrimidines (Figure S1).

### 3.2 Aberrant base misincorporation into DNA caused by dUTPase inhibition led to DNA damage and cell death

To evaluate the DNA damage response in the presence of a dUTPase inhibitor, we monitored PARP activation by measuring the intracellular pool of oxidized NAD (NAD⁺), a substrate of PARP, in HeLa cells. Because PARP is a sensor protein of DNA damage during base excision repair (BER), which is likely to be involved in 5-FU and uracil misincorporation-mediated DNA damage response, and PARP activation in response to DNA damage depletes cellular NAD⁺ levels, 27,28 NAD⁺ is an indicator of PARP activation. Compared with FdUrd alone, the combination of TAS-114 and FdUrd significantly decreased intracellular NAD⁺ levels after 4 h of treatment (Figure 3A).

The three apoptotic markers—cleaved caspase-3, cleaved caspase-9, and cleaved PARP—were detected at substantial levels in HeLa cells after TAS-114 plus FdUrd co-treatment (Figure 3B), but at negligible to very low levels after treatment with FdUrd alone or TAS-114 alone. TS is inhibited by forming a ternary complex with FdUMP and 5,10-methylenetetrahydrofolate, which was detected above the native TS band in western blotting. 29 Considering that the band shift of TS by FdUrd treatment was also detected in the presence of TAS-114 (Figure 3B), TS inhibition was not affected by TAS-114.

Consistent with these findings, over the 48-h period examined, TAS-114 alone had no effect on cell viability, FdUrd suppressed cell growth and slightly decreased viability, and TAS-114 plus FdUrd co-treatment dramatically decreased cell growth and viability after just 24 h of exposure (Figure 3C).
These results suggest that increased misincorporation of aberrant bases, 5-FU and uracil, into DNA as a result of TAS-114-plus-FdUrd co-treatment damaged the DNA, leading to cell death; thus, the cytotoxicity of FdUrd was enhanced in the presence of TAS-114.

3.3 | Deficiency in BER or HR sensitized human cancer cells and a DNA-repair-deficient DT40 cell panel to the combination of TAS-114 and FdUrd

Chicken DT40 cell lines with various gene knockouts related to DNA repair are well established model systems used for drug profiling analysis of DNA-damaging agents.30,31 To investigate which DNA repair pathway contributed to the DNA damage response after dUTPase inhibition, we used a panel of 15 DT40 cell lines (Table S1) to examine the sensitivity to TAS-114-plus-FdUrd co-treatment. In the DT40 cell lines derived from bursal B cells, AID, which triggers immunoglobulin gene diversification, introduces uracil into DNA through cytosine deamination. Hence, to evaluate the impact of UNG deficiency on 5-FU and uracil misincorporation, we used AID- and UNG-deficient (AID–/–UNG–/–) DT40 cell lines.12

The sensitivity profiles of TAS-114-plus-FdUrd in the DT40 cell panel showed different signatures to those of FdUrd alone: compared with the WT cells, the mutant cell lines were generally hypersensitive to the FdUrd-TAS-114 combination, but not to FdUrd alone, FdUrd alone, or TAS-114-plus-FdUrd at the indicated concentrations. Data are means ± SD (n = 3)
(A) FdUrd

|          | WT | ATM | BRCA1 | BRCA2 | PARP | KU70 | PCNA K164R | RAD18 | POLZ | TDP1 | MSH3 | XPA | FEN1 | POLB | AID | AID/UNG |
|----------|----|-----|-------|-------|------|------|-------------|-------|------|------|------|-----|------|------|-----|---------|
| Resistance | -16 | -12 | -8   | -4   | 0    | 4    | 8            | 12   | 16   |

(B) FdUrd + TAS-114

|          | WT | ATM | BRCA1 | BRCA2 | PARP | KU70 | PCNA K164R | RAD18 | POLZ | TDP1 | MSH3 | XPA | FEN1 | POLB | AID | AID/UNG |
|----------|----|-----|-------|-------|------|------|-------------|-------|------|------|------|-----|------|------|-----|---------|
| Resistance | -6  | -4  | -2   | 0    | 2    | 4    | 6            |       |      |      |      |     |      |      |    |         |

(C) FdUrd + TAS-114

![Graphs showing viability against different FdUrd concentrations for various conditions and genotypes.](image-url)
(Figures 4 and S2). This could be attributed to the primary mode of action of FdUrd being the inhibition of cell growth mediated by dTTP depletion, whereas that of TAS-114 in combination with FdUrd is the DNA damage resulting from dTTP depletion (Figure 2). There was no unique signature in DT40 cell lines to paclitaxel, which has a mode of action unrelated to DNA damage (Figure S3).

As expected from the increase in misincorporation of 5-FU and uracil into DNA, DT40 cell lines deficient in the components of the BER pathway, ie, FEN1 and POLB, were hypersensitive to TAS-114-plus-FdUrd co-treatment, and UNG+/−AID+/− cells were more sensitive to the co-treatment compared with AID+/− cells. PARP deficiency also sensitized DT40 cells to TAS-114-plus-FdUrd co-treatment. This finding indicates that PARP has a protective role in HeLa cells treated with TAS-114-plus-FdUrd, which is consistent with the observed decrease in the level of the PARP substrate, NAD+ (Figure 3A). Deficiencies in other DNA repair pathways were also linked to sensitization of the cells to TAS-114-plus-FdUrd co-treatment: eg, deficiency in HR, as demonstrated by deletion or mutation in BRCA1 or BRCA2, and deficiency in translesion DNA synthesis (TLS) as shown by POLZ deletion. In contrast, DT40 cell lines deficient in NER or MMR proteins were relatively resistant to TAS-114-plus-FdUrd co-treatment. This appears to be a general feature of fluoropyrimidine because a similar tendency was observed for FdUrd alone.

To confirm that the hypersensitivity of human cells involves equivalent pathways, as observed in the DT40 chicken lines, we conducted similar experiments by siRNA knockdown of genes related to DNA repair in HeLa cells. Consistent with the results in the chicken cell lines, suppression of BER (ie, deficiency in POLB or UNG) or HR (ie, deficiency in BRCA1 or BRCA2) sensitized HeLa cells to the combination of TAS-114 and FdUrd (Figures 5 and S4).

![Figure 5](image-url)  
**Figure 5** Knockdown of BER or HR pathway genes sensitizes human cancer cells to the combination of TAS-114 with FdUrd. A, Sensitivity profiles for TAS-114 (3 μmol/L)-plus-FdUrd co-treatment in HeLa cells treated with siRNAs against DNA damage repair genes or nonsense siRNA (negative control, NC). Drugs were added 24 h after cell seeding. Thymidine (30 μmol/L) was added 24 h after drug addition, and the cells were incubated for 48 h; except BRCA2 knockdown cells, which were incubated for 96 h. IC_{50} values were calculated, and the sensitivity score for each knockdown cell line was determined relative to negative control cells. B, Viability curve of each knockdown cell line after TAS-114 (3 μmol/L)-plus-FdUrd co-treatment. Data are means ± SD (n = 3). NC, negative control; HR, homologous recombination; MMR, mismatch repair; NER, nucleotide excision repair; BER, base excision repair; BRCA, breast cancer gene; MLH1, mutL homolog 1; ERCC1, ERCC Excision Repair 1; POLB, DNA polymerase beta; UNG, uracil-DNA glycosylase.
Our previous study demonstrated that dUTPase inhibition plays a
crucial role in tumor-selective enhancement of 5-FU-mediated
antiproliferative activity. In this study, we examined the mechanism
of enhancement of antitumor activity underlying dUTPase inhibition;
in particular, we determined the DNA repair pathways that contribute
to the DNA damage response after dUTPase inhibition. Treatment of HeLa cells with FdUrd at 0.1 µmol/L significantly deple-
ted free-TS, indicating TS inhibition. This TS inhibition seemed to
be achieved at near-maximum levels at this concentration be-
cause intracellular dUMP levels were increased by approximately
the same extent that dTTP was depleted at the higher concentra-
tion of 1 µmol/L. Although not marked, FdUrd alone did inhibit cell
proliferation. However, cell death was observed much earlier with
the addition of the dUTPase inhibitor, TAS-114, to FdUrd. RNA incor-
poration is one mechanism of 5-FU-mediated cytotoxicity. However,
because neither the FUTP pool nor 5-FU levels in RNA were
substantially changed by TAS-114-plus-FdUrd co-treatment
in HeLa cells, we concluded that RNA-mediated cytotoxicity may not
be the mechanism behind the potent cell killing caused by dUTP-
ase inhibition. Our observation that a DNA damage response (ie, NAD+ depletion) was observed after TAS-114-plus-FdUrd co‐treatment indicated that aberrant base misincorporation leads to DNA
damage, resulting in cell death. The fluoropyrimidine metabolite, FdUMP, irreversibly binds to TS and inhibits its ability to convert
dUMP to dTMP. TS inhibition leads to dTTP depletion and impedes cell proliferation by starving the cells of substrates essential for
DNA polymerization. Conversely, TS inhibition-plus-dUTPase in-
hibition induces a more unbalanced nucleotide pool and supplies alternative DNA substrates such as dUTP and FdUTP instead of
dTTP. This probably facilitates cell cycle progression despite the
absence of dTTP and eventually results in severe DNA damage fol-
lowed by cell death.

Our analysis of the relationship between DNA repair pathways
and drug sensitivity in the DT40 cell panel and the knockdown ex-
periments in HeLa cells suggest that both BER and HR are respon-
sible for the DNA damage response mediated by dUTPase inhibition,
and that these DNA repair pathways directly affect the sensitivity
of TAS-114 in combination with FdUrd. BER acts as a primary DNA
repair system by removing aberrant bases in DNA. Therefore, it is
plausible that BER would be involved in DNA damage repair activ-
ated by 5-FU and uracil misincorporation due to dUTPase inhibi-
tion. UNG is a DNA glycosylase that is primarily responsible for
removing 5-FU and uracil misincorporated in DNA. We found that
deficiency or suppression of UNG caused a dramatic increase in the
cytotoxicity of TAS-114 in combination with FdUrd, indicating that
massive misincorporation of uracil and 5-FU into DNA was toxic
to cancer cells. The 5-FU-adenine base pair is relatively unstable
compared with thymine-adenine, therefore the lower stability
caued by 5-FU and uracil misincorporation may lead to general
DNA dysfunction and subsequent cell death. POLZ catalyzes the removal of 5'-deoxyribose phosphate along with gap-filling DNA
synthesis in the short-pathway; FEN1 recognizes and cleaves
5'-single-stranded DNA flaps in the long-pathway, and pu-
rinic/apyrimidinic endonuclease 1 (APE1) cleaves the abasic (AP)
sites generated when DNA glycosylase removes the damaged bases
during the process of BER. When POLB or FEN1 were suppressed or
deficient, numerous DNA nicks generated by APE1 were probably
not repaired, which enhanced the cytotoxicity of dUTPase inhibitor.

TAS-114 alone decreased intracellular NAD+ levels in HeLa cells.
Our findings suggested that TAS-114 enhanced intrinsic uracil incor-
poration into DNA and caused DNA damage at higher concen-
trations, however its effect on cell growth seemed to be minimal in
the absence of fluoropyrimidines. Thus, it has no obvious intrinsic
antiproliferative activity. These results indicated that induction of
cytotoxicity requires substantial amounts of 5-FU and uracil incor-
poration into DNA.

BRCAs are key components of HR, which is functionally cru-
cial for the accurate repair of DNA double-stranded breaks. Interestingly, not only DNA repair genes for single-stranded breaks,
but also those for double-stranded breaks, appeared to be involved
in the repair process after 5-FU and uracil misincorporation by TAS-
114-plus-FdUrd co-treatment. When massive amounts of 5-FU and
uracil are incorporated into DNA, the BER capacity is overwhelmed,
potentially leading to secondary DNA double-stranded breaks. When dUTPase is inhibited, DNA double-stranded break repair may be a back-up mechanism after BER fails.

Deficiency of BER or HR genes also slightly sensitized DT40 cells
to FdUrd alone, as observed in previous studies, although the magnitude of the differences was much smaller compared with that observed for the FdUrd-TAS-114 combination. This is because the primary mode of action of FdUrd is inhibition of cell growth medi-
ated by dTTP depletion, and 5-FU and uracil misincorporation into
DNA is limited by dUTPase.

One mechanism of cell death after 5-FU treatment is the fu-
tile cyclic removal and incorporation of 5-FU paired with guanine by
the mismatch repair pathway involving MSH3 and MLH1. Here, MSH3 deficiency in DT40 cells and MLH1 suppression in HeLa
cells both decreased sensitivity to the FdUrd-TAS-114 combina-
tion. This was also observed in DT40 cell lines treated with FdUrd
alone. Therefore, we hypothesized that mechanisms of DNA repair in
5-FU-guanine base pairs would be independent from dUTPase in-
hibitor-mediated cytotoxicity.

POLZ is responsible for DNA polymerization in TLS, and abasic sites are bypassed by the DNA polymerases responsible for
TLS. Increase in 5-FU and uracil misincorporation into DNA
may increase the number of abasic sites during DNA repair, and
TLS may play a role in tolerance for aberrant base misincor-
poration-induced cell death by extending the DNA strand opposite to the abasic sites. POLZ deficiency sensitized the DT40 cells to TAS-
114-plus-FdUrd, whereas the PCNAK168 mutation does not affect the
cytotoxicity of this combination. Although POLZ and PCNA
seem to work together in DT40 cells, because the sensitivity to DNA damage agents such as cisplatin increases equally in the
POLZ−/− cells and PCNA−/− cells, our results suggested that only
POLZ appears to be involved in the antitumor activity of the TASS-114-plus-FdUrd combination. Further study is required to explore other non-TLS mechanisms that could explain the antitumor activity of dUTPase inhibition.

In summary, this study demonstrates that the BER and HR pathways play substantial roles in DNA repair when dUTPase is inhibited in the presence of FdUrd, a TS inhibitor. TASS-114 also enhances the antitumor activity of pemetrexed, probably by enhancing DNA damage. Because pemetrexed is a non-fluoropyrimidine TS inhibitor, co-treatment with TASS-114 potentiated the activity of pemetrexed by enhancing the misincorporation of only uracil. Although the difference in DNA repair pathways should be explored for 5-FU and/or uracil misincorporation, DNA lesions induced by misincorporation of aberrant bases are recognized and repaired by both BER and HR, and deficiencies in these pathways contribute to the enhanced cytotoxicity caused by dUTPase-inhibitor-plus-fluoropyrimidine compared with fluoropyrimidine alone. These findings support the hypothesis that DNA-repair-defective cancers, such as BRCA-deficient cancers, could be promising targets of dUTPase-inhibitor-plus-fluoropyrimidine combination therapy.

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DISCLOSURE STATEMENT

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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