Received: 1 July 2020 | Revised: 28 October 2020 | Accepted: 30 October 2020

DOI: 10.1111/cas.14718

ORIGINAL ARTICLE

Cancer Science WILEY

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

Tatsushi Yokogawa | Wakako Yano | Sayaka Tsukioka | Akiko Osada | Takeshi Wakasa | Hiroyuki Ueno | Takuya Hoshino | Keisuke Yamamura | Akio Fujioka | Masayoshi Fukuoka | Shuichi Ohkubo | Kenichi Matsuo

Discovery and Preclinical Research Division, Taiho Pharmaceutical Co., Ltd., Tsukuba, Japan

**Correspondence**
Shuichi Ohkubo, Taiho Pharmaceutical Co., Ltd., 3 Okubo, Tsukuba, Ibaraki 300-2611, Japan.
Email: s-ohkubo@taiho.co.jp

**Funding information**
Taiho Pharmaceutical Co., Ltd.

## 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.[1] An underlying mechanism of carcinogenesis is abnormality or deficiency in DNA repair, as evidenced by DNA mismatch repair (MMR) deficiency in colorectal cancer[2] and homologous recombination repair (HR) deficiency because of breast cancer susceptibility gene (BRCA1/2) mutations in breast and ovarian cancers.[3] 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.[4,5]

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.[6,7] $O^6$-Methylguanine-DNA methyltransferase plays a crucial role in the removal of modified bases in DNA necessary for the antitumor activity of alkylating agents.[8]

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.[9,10] Although uracil is not a component of DNA, DNA polymerase can utilize dTTP and dUTP with equal efficiency in DNA synthesis.[11] Therefore, dUTPase maintains the intracellular dUTP pool at an extremely low level to minimize uracil misincorporation into DNA.[12,13]

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.[14] 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.[15,16] 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;[9,15-18] hence, dUTPase is a potential target for improving this chemotherapy's efficacy.[10,19]

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.[20] 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.[21,22] 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*-[(1*R*)-1-[3-(cyclopentyloxy)phenyl]-ethyl]-3-[(3,4-dihydro-2,4-dioxo-1(2*H*)-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 Carbosynth Limited. [6-$^3$H]-FdUMP (666 GBq/mmol), [$^3$H]-FdUrd (614 GBq/mmol), and [$^3$H]-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

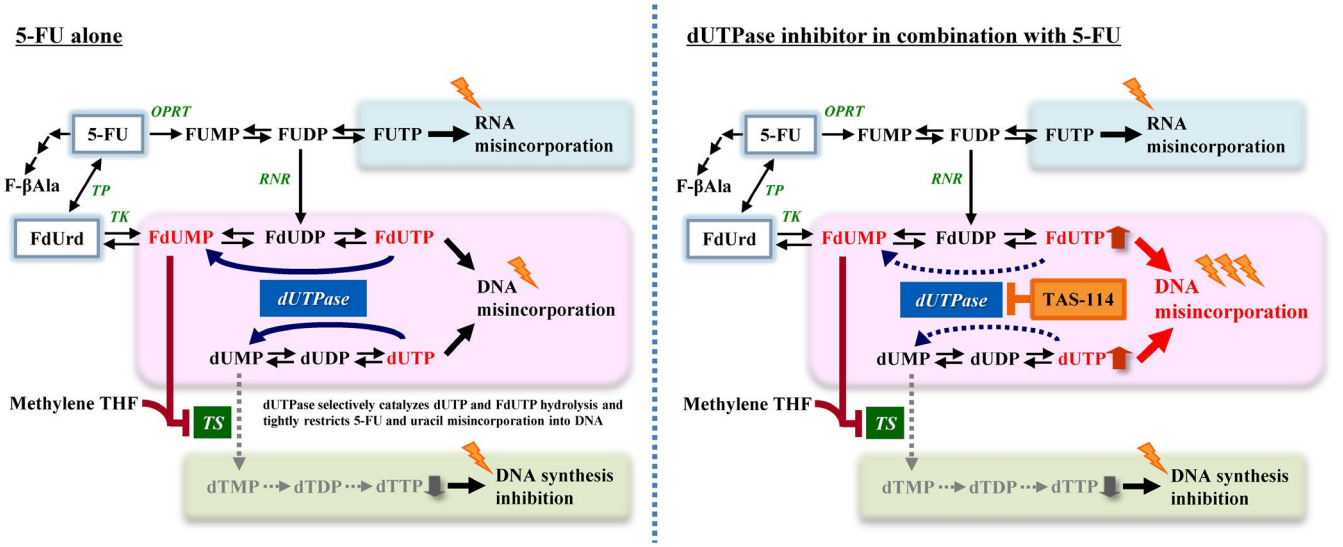



**FIGURE 1** Mechanism of action of dUTPase inhibitor, TAS-114, in combination with 5-FU. TAS-114 inhibition of dUTPase in cancer cells facilitates the incorporation of FdUTP and dUTP into DNA, thereby enhancing the cytotoxicity of fluoropyrimidine. F-βAla, 2-fluoro-β-alanine; FdUDP, 5-fluoro-2′-deoxyuridine 5′-diphosphate; FUMP, 5-fluorouridine 5'-monophosphate; FUDP, 5-fluorouridine 5'-diphosphate; methylene THF, 5,10-methylenetetrahydrofolate

were kindly provided by Shunichi Takeda (Kyoto University) and Patricia Gearhart (National Institute on Aging).

## 2.3 | Measurement of intracellular nucleotide pool

HeLa cells ($5 \times 10^6$ cells) were seeded into 175-cm$^2$ 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.[20] The dUTP levels were measured using a PCR thermocycler-based fluorescence-based assay developed by Wilson et al.[23]

## 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;[24] see our previous report.[20] Free-TS levels per $10^6$ cells were calculated relative to the total protein levels.

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

HeLa cells were seeded into 75-cm$^2$ flasks ($2 \times 10^6$ cells) and in 6-well plates ($2.5 \times 10^5$ 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 [$^3$H]-5-FU per μg of DNA or RNA were calculated.

## 2.6 | Measurement of cell viability

HeLa cells were seeded into 12-well plates ($5 \times 10^4$ 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 ($2 \times 10^5$ 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.). $IC_{50}$ 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 = $\log_2(Y/X)$, where Y = $IC_{50}$ of mutant cells and X = $IC_{50}$ 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 ($1 \times 10^5$ cells) were seeded into 75-cm$^2$ 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 $2 \times 10^3$ 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. Cellular proliferation was evaluated by crystal violet staining, as described previously.[20] $IC_{50}$ 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 = $\log_2(Y/X)$, where Y = $IC_{50}$ of knockdown cells and X = $IC_{50}$ 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-methylenetetrahydrofolate is responsible for FdUMP-mediated inhibition of TS.[25] 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 dTTP compared to those with FdUrd alone.

Fluoropyrimidines are metabolized not only to FdUMP, but also to ribonucleotides, which cause RNA-based cytotoxicity.[26] 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.

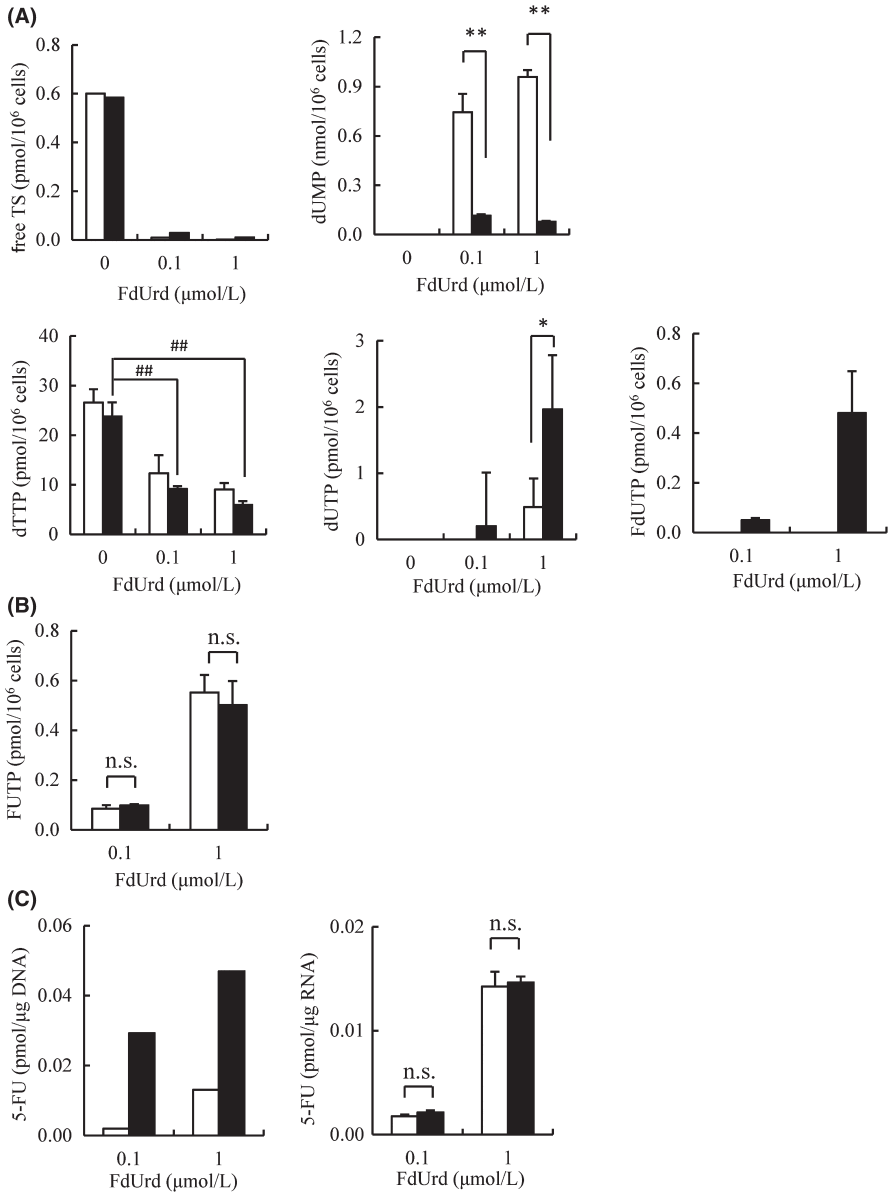



**FIGURE 2** TAS-114-plus-FdUrd co-treatment inhibited dUTPase and facilitated aberrant base misincorporation into DNA in HeLa cells. HeLa cells were exposed to FdUrd alone at 0, 0.1, or 1 μmol/L (□) or in combination with TAS-114 (10 μmol/L) (■) for 8 h, and the levels of (A) intracellular TS (free-TS) ($n = 1$), dUMP, dTTP, dUTP, and FdUTP ($n = 3$), (B) FUTP ($n = 3$), and (C) 5-FU in DNA ($n = 2$) and RNA ($n = 3$) were measured. Data are means ± SD; no error bars are displayed for $n \leq 2$. *, $P < 0.05$ and **, $P < 0.01$ vs. FdUrd alone. ##, $P < 0.01$ vs. TAS-114 alone. n.s., not significant

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).

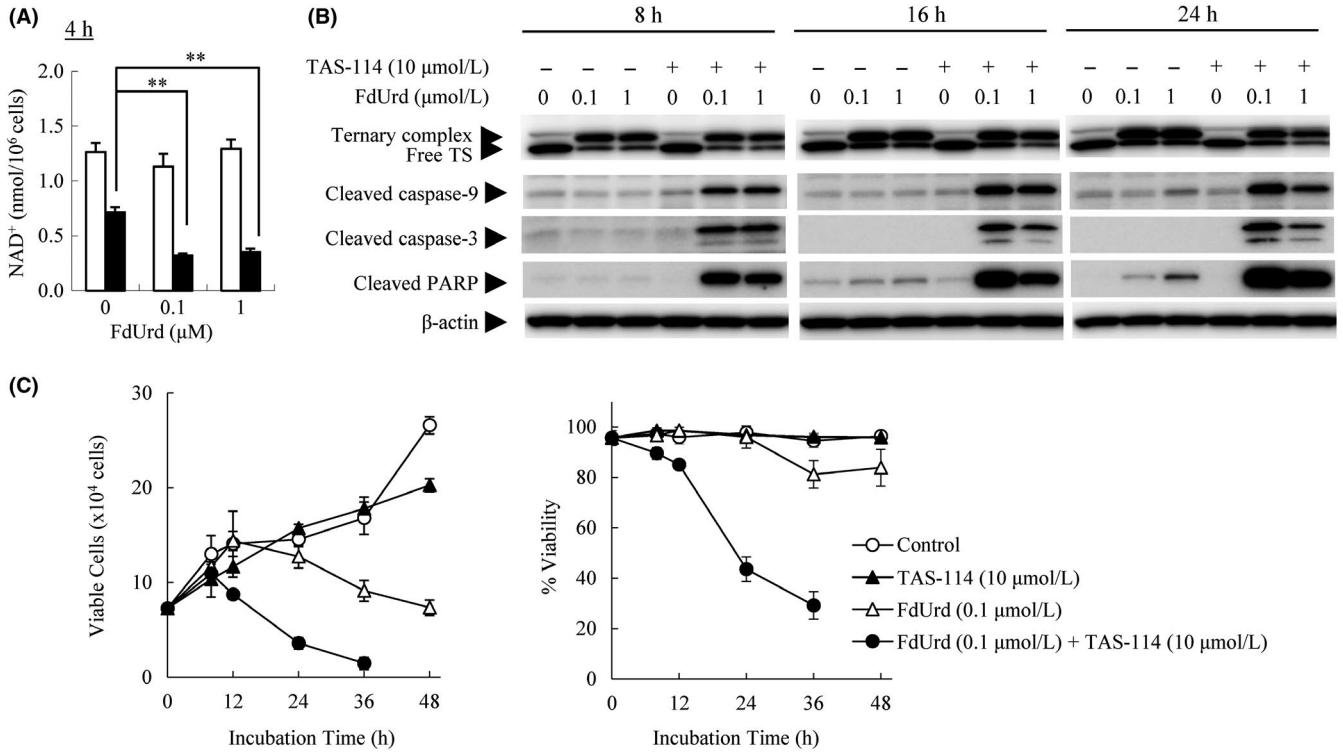



**FIGURE 3** DNA damage response and induction of cell death by TAS-114-enhanced FdUrd cytotoxicity in HeLa cells. A, HeLa cells were exposed to FdUrd alone at 0, 0.1, or 1 μmol/L (□) or in combination with TAS-114 (10 μmol/L) (■) for 4 h, and the intracellular $NAD^+$ levels were measured. Data are means ± SD ($n = 3$). **, $P < 0.01$ vs. TAS-114 alone. B, Effect of TAS-114-plus-FdUrd on cell death markers. HeLa cells were exposed to FdUrd at 0, 0.1, or 1 μmol/L with or without TAS-114 at 10 μmol/L for 8, 16, or 24 h, and the levels of the indicated apoptotic marker proteins were measured: cleaved caspase-3, cleaved caspase-9, and cleaved PARP. To evaluate TS inhibition, the band shift of TS due to the formation of ternary complex was evaluated. β-Actin was used as a loading control. C, Time course in cell growth and survival rates of HeLa cells were measured after administration of TAS-114 alone, FdUrd alone, or TAS-114-plus-FdUrd at the indicated concentrations. Data are means ± SD ($n = 3$)

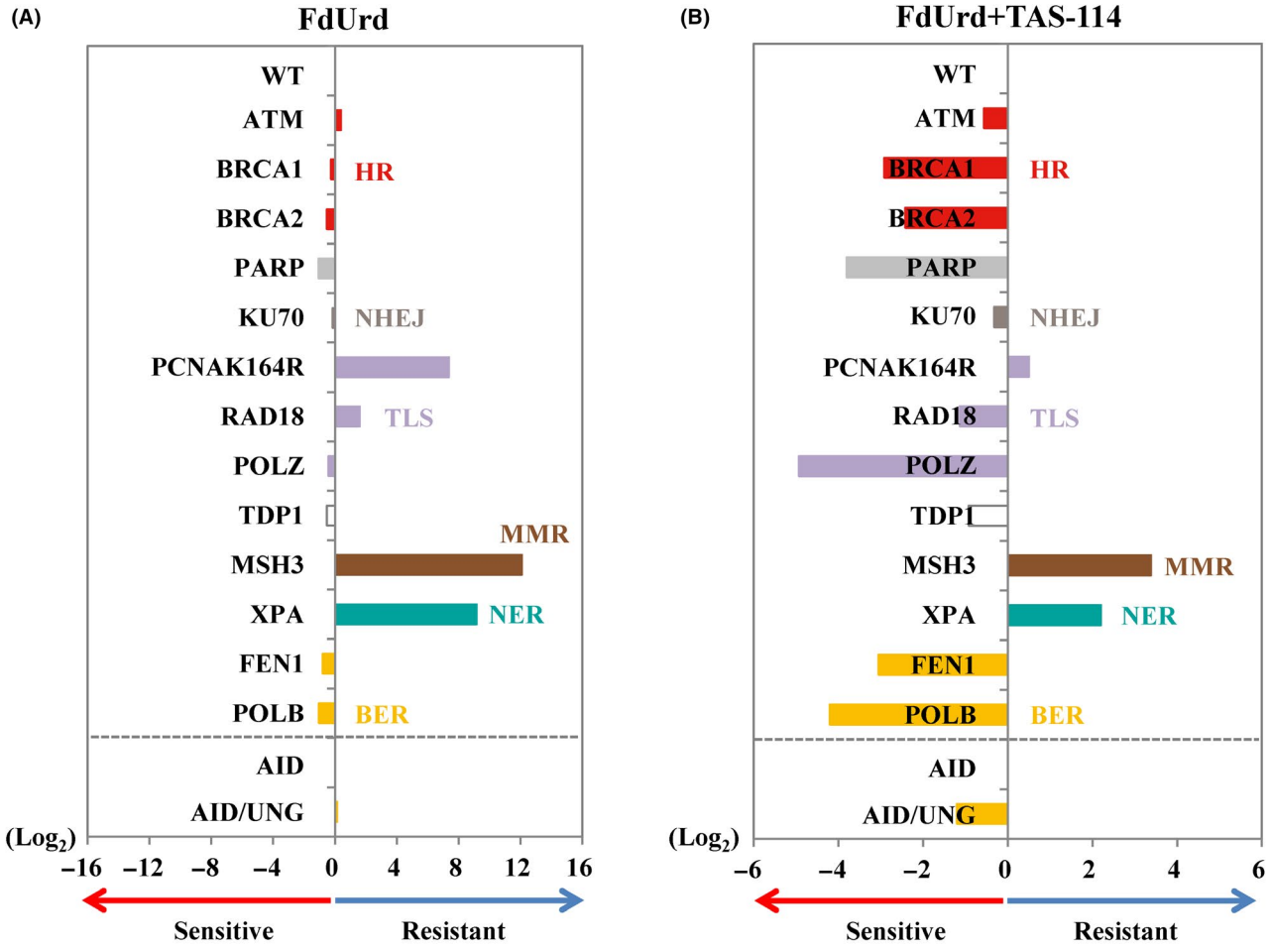
These results suggest that increased misincorporation of aberrant bases, 5-FU and uracil, into DNA as a result of TAS-114-plus-FdUrd co-treatment damaged the DNA, leading to cell death; thus, the cytotoxicity of FdUrd was enhanced in the presence of TAS-114.

### 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

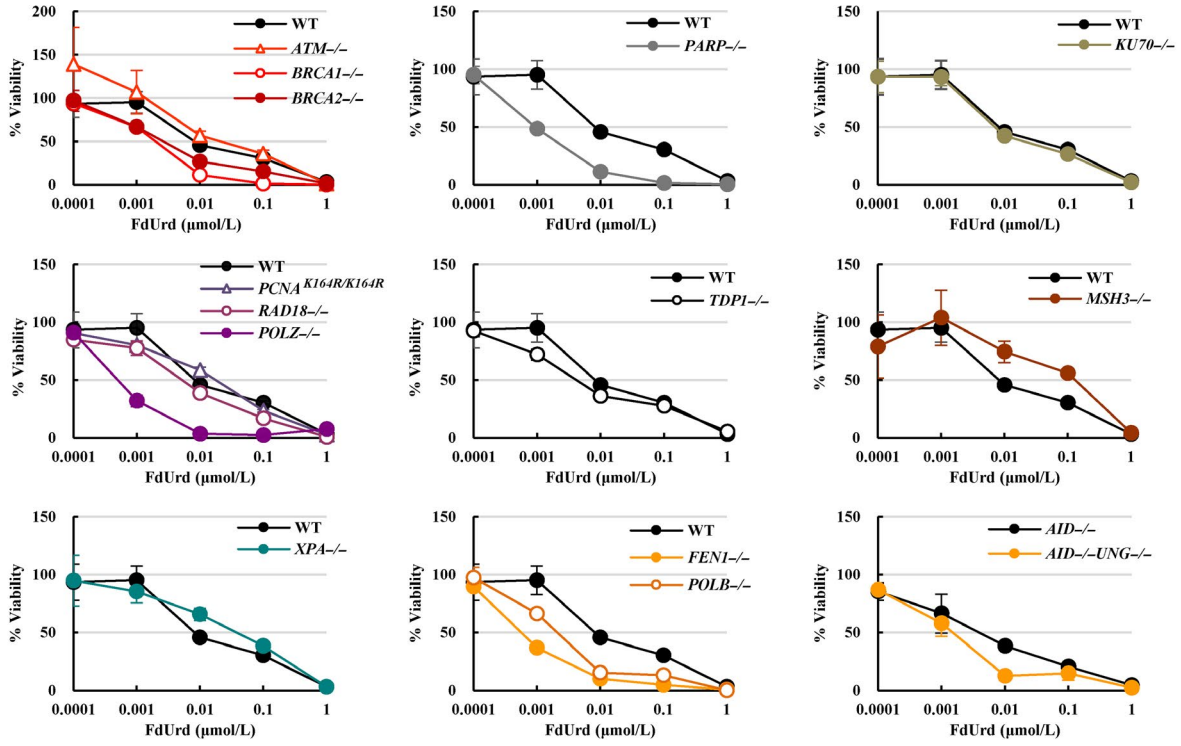
Chicken DT40 cell lines with various gene knockouts related to DNA repair are well established model systems used for drug profiling analysis of DNA-damaging agents.[30,31] To investigate which DNA repair pathway contributed to the DNA damage response after dUTPase inhibition, we used a panel of 15 DT40 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

**FIGURE 4** Role of DNA damage repair pathways in the cytotoxicity of the combination treatment with TAS-114 and FdUrd. A, Sensitivity profiles for FdUrd alone or TAS-114 (1 μmol/L)-plus-FdUrd co-treatment in DNA-repair-deficient DT40 cell panel. Drugs were added, and the cells were incubated for 72 h. $IC_{50}$ values for all cell lines were calculated, and the sensitivity score for each mutant cell line was determined relative to wild-type cells, except the score in the $AID^{-/-}UNG^{-/-}$ cell line, which was calculated relative to the $AID^{-/-}$ cell line (see Materials and Methods). B, Viability curves of mutant DT40 cell lines after TAS-114 (1 μmol/L)-plus-FdUrd co-treatment for 72 h. Data are means ± SD ($n = 3$). WT, wild-type; HR, homologous recombination; NHEJ, non-homologous end joining; TLS, translesion DNA synthesis; MMR, mismatch repair; NER, nucleotide excision repair; BER, base excision repair; ATM, Ataxia telangiectasia mutated; BRCA, breast cancer gene; PARP1, poly (ADP-ribose) polymerase 1; KU70, ATP-dependent DNA helicase 2 subunit 1; RAD18, E3 protein ubiquitin ligase; POLZ, DNA polymerase zeta; TDP1, tyrosyl-phosphodiesterase 1; MSH3, mutS homolog 3; XPA, DNA damage recognition and repair factor; FEN1, Flap endonuclease 1; POLB, DNA polymerase beta; AID, activation-induced cytidine deaminase; UNG, uracil-DNA glycosylase




(C) **FdUrd + TAS-114**



(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 5-FU and uracil misincorporation (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).

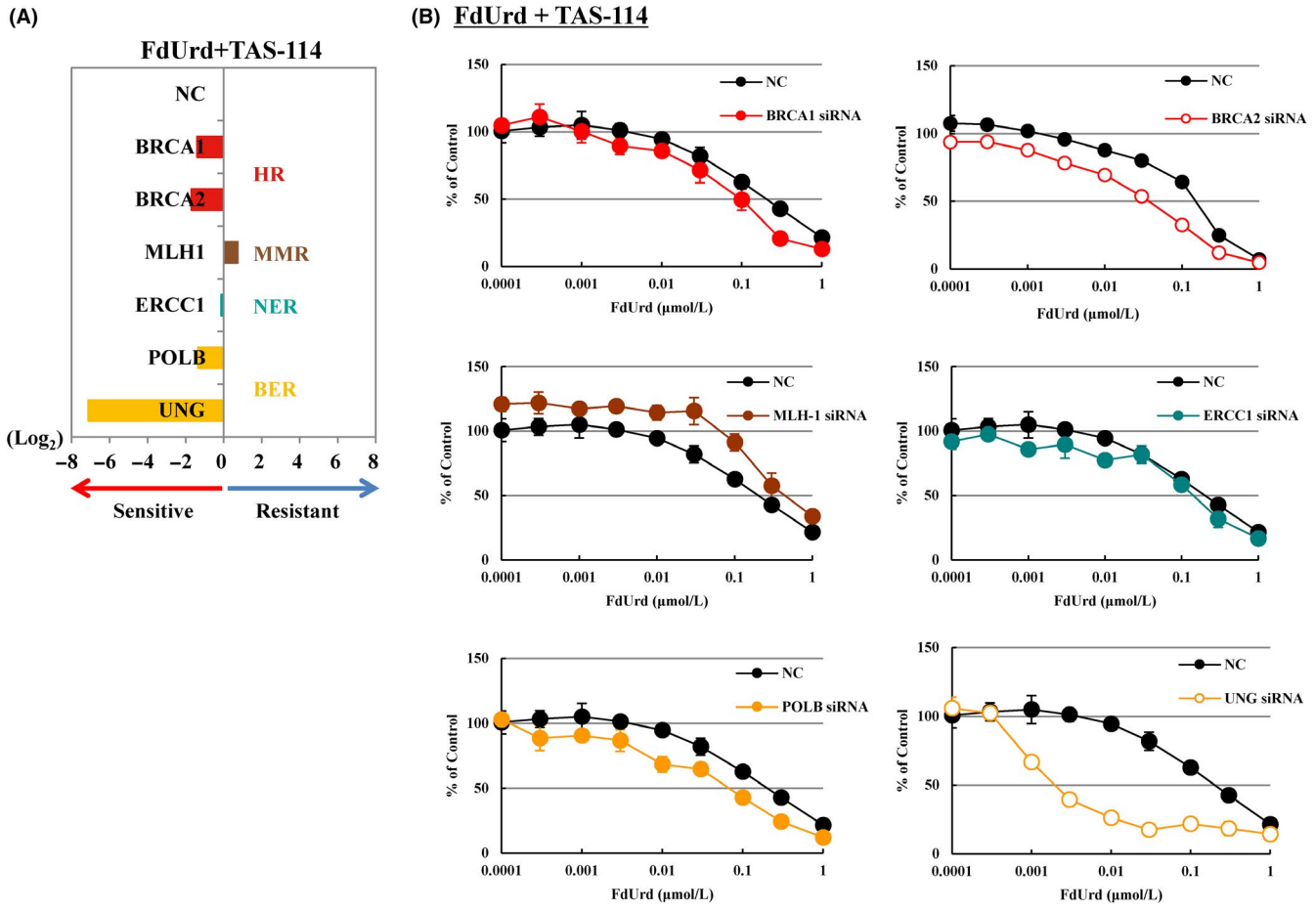



**FIGURE 5** Knockdown of BER or HR pathway genes sensitizes human cancer cells to the combination of TAS-114 with FdUrd. A, Sensitivity profiles for TAS-114 (3 μmol/L)-plus-FdUrd co-treatment in HeLa cells treated with siRNAs against DNA damage repair genes or nonsense siRNA (negative control, NC). 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

## 4 | DISCUSSION

Our previous study demonstrated that dUTPase inhibition plays a crucial role in tumor-selective enhancement of 5-FU-mediated antiproliferative activity.[20] 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 depleted free-TS, indicating TS inhibition. This TS inhibition seemed to be achieved at near-maximum levels at this concentration because intracellular dUMP levels were increased by approximately the same extent that dTTP was depleted at the higher concentration 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 incorporation is one mechanism of 5-FU-mediated cytotoxicity.[26,32] 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 dUTPase 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.[32] Conversely, TS inhibition-plus-dUTPase inhibition 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 followed by cell death.

Our analysis of the relationship between DNA repair pathways and drug sensitivity in the DT40 cell panel and the knockdown experiments in HeLa cells suggest that both BER and HR are responsible 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 activated by 5-FU and uracil misincorporation due to dUTPase inhibition. UNG is a DNA glycosylase that is primarily responsible for removing 5-FU and uracil misincorporated in DNA.[33] 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,[34,35] therefore the lower stability caused by 5-FU and uracil misincorporation may lead to general DNA dysfunction and subsequent cell death.[36,37] POLB catalyzes the removal of 5′-deoxyribose phosphate along with gap-filling DNA synthesis in the short-patch pathway; FEN1 recognizes and cleaves 5′-single-stranded DNA flaps in the long-patch pathway;[33] and purinic/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 incorporation into DNA and caused DNA damage at higher concentrations, 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 incorporation into DNA.

BRCAs are key components of HR, which is functionally crucial for the accurate repair of DNA double-stranded breaks.[38] 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.[39] 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,[21,22] 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 mediated 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 futile cyclic removal and incorporation of 5-FU paired with guanine by the mismatch repair pathway involving MSH3 and MLH1.[40,41] Here, *MSH3* deficiency in DT40 cells and *MLH1* suppression in HeLa cells both decreased sensitivity to the FdUrd-TAS-114 combination. 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 inhibitor-mediated cytotoxicity.

POLZ is responsible for DNA polymerization in TLS,[42-45] and abasic sites are bypassed by the DNA polymerases responsible for TLS.[46,47] 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 misincorporation-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 $PCNA^{K16R}$ 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,[48] our results suggested that only

POLZ appears to be involved in the antitumor activity of the TAS-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. TAS-114 also enhances the antitumor activity of pemetrexed, probably by enhancing DNA damage.[49] Because pemetrexed is a non-fluoropyrimidine TS inhibitor, co-treatment with TAS-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 supported the hypothesis that DNA-repair-defective cancers, such as BRCA-deficient cancers, could be promising targets of dUTPase-inhibitor-plus-fluoropyrimidine combination therapy.

## ACKNOWLEDGMENTS

All research was funded by Taiho Pharmaceutical Co., Ltd. The authors thank Junko Murai (National Cancer Institute, Bethesda), James T. Stivers (Johns Hopkins Medicine, Baltimore), and Teruhiro Utsugi (Taiho Pharmaceuticals, Tokyo) for their insightful discussions. We thank Shunichi Takeda (Kyoto University, Kyoto) for the DT40 cell lines and Patricia Gearhart (National Institute on Aging, Baltimore) for the DT40 *AID*$^{-/-}$ and DT40 *AID*$^{-/-}$*UNG*$^{-/-}$ cell lines.

## DISCLOSURE STATEMENT

All authors are full-time employees of Taiho Pharmaceutical Co., Ltd.

## ORCID

*Shuichi Ohkubo* https://orcid.org/0000-0001-9554-4534

## REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
2. Boland CR, Goel A. Microsatellite instability in colorectal cancer. *Gastroenterology*. 2010;138:2073-2087.
3. Lord CJ, Ashworth A. BRCAness revisited. *Nat Rev Cancer*. 2016;16:110-120.
4. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*. 2005;434:913-917.
5. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921.
6. Lord RV, Brabender J, Gandara D, et al. Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. *Clin Cancer Res*. 2002;8:2286-2291.
7. Selvakumaran M, Pisarcik DA, Bao R, et al. Enhanced cisplatin cytotoxicity by disturbing the nucleotide excision repair pathway in ovarian cancer cell lines. *Cancer Res*. 2003;63:1311-1316.
8. Drablos F, Feyzi E, Aas PA, et al. Alkylation damage in DNA and RNA–repair mechanisms and medical significance. *DNA Repair (Amst)*. 2004;3:1389-1407.
9. Ladner RD, Lynch FJ, Groshen S, et al. dUTP nucleotidohydrolase isoform expression in normal and neoplastic tissues: association with survival and response to 5-fluorouracil in colorectal cancer. *Cancer Res*. 2000;60:3493-3503.
10. Wilson PM, Danenberg PV, Johnston PG, et al. Standing the test of time: targeting thymidylate biosynthesis in cancer therapy. *Nature Rev Clin Oncol*. 2014;11:282-298.
11. Aherne GW, Brown S. The role of uracil misincorporation in thymineless death. In: Jackman AL, ed. *Antifolate Drugs in Cancer Therapy*. Totowa, NJ: Humana Press; 1999:409-421.
12. Grogan BC, Parker JB, Guminski AF, et al. Effect of the thymidylate synthase inhibitors on dUTP and TTP pool levels and the activities of DNA repair glycosylases on uracil and 5-fluorouracil in DNA. *Biochemistry*. 2011;50:618-627.
13. Traut TW. Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem*. 1994;140:1-22.
14. van Laar JA, van der Wilt CL, Rustum YM, et al. Therapeutic efficacy of fluoropyrimidines depends on the duration of thymidylate synthase inhibition in the murine colon 26-B carcinoma tumor model. *Clin Cancer Res*. 1996;2:1327-1333.
15. Wilson PM, Fazzone W, LaBonte MJ, et al. Novel opportunities for thymidylate metabolism as a therapeutic target. *Mol Cancer Ther*. 2008;7:3029-3037.
16. Wilson PM, LaBonte MJ, Lenz HJ, et al. Inhibition of dUTPase induces synthetic lethality with thymidylate synthase-targeted therapies in non-small cell lung cancer. *Mol Cancer Ther*. 2012;11:616-628.
17. Kawahara A, Akagi Y, Hattori S, et al. Higher expression of deoxyuridine triphosphatase (dUTPase) may predict the metastasis potential of colorectal cancer. *J Clin Pathol*. 2009;62:364-369.
18. Takatori H, Yamashita T, Honda M, et al. dUTP pyrophosphatase expression correlates with a poor prognosis in hepatocellular carcinoma. *Liver Int*. 2010;30:438-446.
19. Peters GJ. Novel developments in the use of antimetabolites. *Nucleosides Nucleotides Nucleic Acids*. 2014;33:358-374.
20. Yano W, Yokogawa T, Wakasa T, et al. TAS-114, a first-in-class dual dUTPase/DPD inhibitor, demonstrates potential to improve therapeutic efficacy of fluoropyrimidine-based chemotherapy. *Mol Cancer Ther*. 2018;17:1683-1693.
21. Huehls AM, Huntoon CJ, Joshi PM, et al. Genomically incorporated 5-fluorouracil that escapes UNG-initiated base excision repair blocks DNA replication and activates homologous recombination. *Mol Pharmacol*. 2016;89:53-62.
22. Huehls AM, Wagner JM, Huntoon CJ, et al. Identification of DNA repair pathways that affect the survival of ovarian cancer cells treated with a poly(ADP-ribose) polymerase inhibitor in a novel drug combination. *Mol Pharmacol*. 2012;82:767-776.
23. Wilson PM, Labonte MJ, Russell J, et al. A novel fluorescence-based assay for the rapid detection and quantification of cellular deoxyribonucleoside triphosphates. *Nucleic Acids Res*. 2011;39:e112.
24. Takeda S, Uchida J, Yamada Y, et al. The significance of measuring inhibition of thymidylate synthase activity as a parameter for antitumor activity of 5-fluorouracil derivatives. *Gan To Kagaku Ryoho*. 1988;15:2125-2130.
25. Tsukioka S, Uchida J, Tsujimoto H, et al. Oral fluoropyrimidine S-1 combined with leucovorin is a promising therapy for colorectal cancer: Evidence from a xenograft model of folate-depleted mice. *Mol Med Rep*. 2009;2:393-398.
26. Burger K, Muhl B, Harasim T, et al. Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. *J Biol Chem*. 2010;285:12416-12425.

27. Zong WX, Ditsworth D, Bauer DE, et al. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev*. 2004;18:1272-1282.
28. Alano CC, Ying W, Swanson RA. Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires $NAD^+$ depletion and mitochondrial permeability transition. *J Biol Chem*. 2004;279:18895-18902.
29. Drake JC, Allegra CJ, Johnston PG. Immunological quantitation of thymidylate synthase-FdUMP-5,10-methylenetetrahydrofolate ternary complex with the monoclonal antibody TS 106. *Anticancer Drugs*. 1993;4:431-435.
30. Maede Y, Shimizu H, Fukushima T, et al. Differential and common DNA repair pathways for topoisomerase I- and II-targeted drugs in a genetic DT40 repair cell screen panel. *Mol Cancer Ther*. 2014;13:214-220.
31. Murai J, Huang SY, Das BB, et al. Trapping of PARP1 and PARP2 by clinical PARP inhibitors. *Cancer Res*. 2012;72:5588-5599.
32. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nature Rev Cancer*. 2003;3:330-338.
33. Krokan HE, Drabløs F, Slupphaug G. Uracil in DNA–occurrence, consequences and repair. *Oncogene*. 2002;21:8935-8948.
34. Kremer AB, Mikita T, Beardsley GP. Chemical consequences of incorporation of 5-fluorouracil into DNA as studied by NMR. *Biochemistry*. 1987;26:391-397.
35. Parker JB, Stivers JT. Dynamics of uracil and 5-fluorouracil in DNA. *Biochemistry*. 2011;50:612-617.
36. el-Hajj HH, Wang L, Weiss B. Multiple mutant of *Escherichia coli* synthesizing virtually thymineless DNA during limited growth. *J Bacteriol*. 1992;174:4450-4456.
37. Gadsden MH, McIntosh EM, Game JC, et al. dUTP pyrophosphatase is an essential enzyme in *Saccharomyces cerevisiae*. *EMBO J*. 1993;12:4425-4431.
38. Curtin NJ. DNA repair dysregulation from cancer driver to therapeutic target. *Nature Rev Cancer*. 2012;12:801-817.
39. Berger SH, Pittman DL, Wyatt MD. Uracil in DNA: consequences for carcinogenesis and chemotherapy. *Biochem Pharmacol*. 2008;76:697-706.
40. Meyers M, Wagner MW, Hwang HS, et al. Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. *Cancer Res*. 2001;61:5193-5201.
41. Meyers M, Wagner MW, Mazurek A, et al. DNA mismatch repair-dependent response to fluoropyrimidine-generated damage. *J Biol Chem*. 2005;280:5516-5526.
42. Lawrence CW. Cellular roles of DNA polymerase zeta and Rev1 protein. *DNA Repair (Amst)*. 2002;1:425-435.
43. Xie Z, Braithwaite E, Guo D, et al. Mutagenesis of benzo[a]pyrene diol epoxide in yeast: requirement for DNA polymerase zeta and involvement of DNA polymerase eta. *Biochemistry*. 2003;42:11253-11262.
44. Zhao B, Wang J, Geacintov NE, et al. Poleta, Polzeta and Rev1 together are required for G to T transversion mutations induced by the (+)- and (-)-trans-anti-BPDE-N2-dG DNA adducts in yeast cells. *Nucleic Acids Res*. 2006;34:417-425.
45. Sale JE. Competition, collaboration and coordination–determining how cells bypass DNA damage. *J Cell Sci*. 2012;125:1633-1643.
46. Haracska L, Unk I, Johnson RE, et al. Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites. *Genes Dev*. 2001;15:945-954.
47. Shachar S, Ziv O, Avkin S, et al. Two-polymerase mechanisms dictate error-free and error-prone translesion DNA synthesis in mammals. *EMBO J*. 2009;28:383-393.
48. Bruno PM, Liu Y, Park GY, et al. A subset of platinum-containing chemotherapeutic agents kills cells by inducing ribosome biogenesis stress. *Nat Med*. 2017;23:461-471.
49. Yano W, Tsukioka S, Yokogawa T, et al. TAS-114, a dUTPase inhibitor, in combination with pemetrexed is a novel strategy for the treatment of NSCLC. *Mol Cancer Ther*. 2013;12:B88.


## SUPPORTING INFORMATION

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

**How to cite this article:** Yokogawa T, Yano W, Tsukioka S, et al. dUTPase inhibition confers susceptibility to a thymidylate synthase inhibitor in DNA-repair-defective human cancer cells. *Cancer Sci*. 2021;112:422–432. https://doi.org/10.1111/cas.14718