TAS-114, a First-in-Class Dual dUTPase/DPD Inhibitor, Demonstrates Potential to Improve Therapeutic Efficacy of Fluoropyrimidine-based Chemotherapy

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Abstract

5-Fluorouracil (5-FU) is an antimetabolite and exerts antitumor activity via intracellularly and physiologically complicated metabolic pathways. In this study, we designed a novel small molecule inhibitor, TAS-114, which targets the intercellular metabolism of 5-FU to enhance antitumor activity and modulates catabolic pathway to improve the systemic availability of 5-FU.

TAS-114 strongly and competitively inhibited deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase), a gatekeeper protein preventing aberrant base incorporation into DNA, and enhanced the cytotoxicity of fluoropyrimidines in cancer cells; however, it had little intrinsic activity. In addition, TAS-114 had moderate and reversible inhibitory activity on dihydropyrimidine dehydrogenase (DPD), a catabolizing enzyme of 5-FU. Thus, TAS-114 increased the bioavailability of 5-FU when co-administered with capecitabine in mice, and it significantly improved the therapeutic efficacy of capecitabine by reducing the required dose of the prodrug by dual enzyme inhibition. Enhancement of antitumor efficacy caused by the addition of TAS-114 was retained in the presence of a potent DPD inhibitor containing oral fluoropyrimidine (S-1), indicating that dUTPase inhibition plays a major role in enhancing the antitumor efficacy of fluoropyrimidine-based therapy.

In conclusion, TAS-114, a dual dUTPase/DPD inhibitor, demonstrated the potential to improve the therapeutic efficacy of fluoropyrimidine. Dual inhibition of dUTPase and DPD is a novel strategy for the advancement of oral fluoropyrimidine-based chemotherapy for cancer treatment.
Introduction

Recently developed cancer treatments, such as targeting driver gene mutations in cancer and immune checkpoint blockade, have impressively improved the prognosis of cancer patients. Nevertheless, traditional chemotherapy remains the cornerstone of cancer treatment. 5-Fluorouracil (5-FU) has been used for more than 50 years since its discovery (1, 2), and new strategies have been developed to increase its anticancer activity, including addition of a modulator, development of a prodrug, and combination therapy (3, 4). Several 5-FU-based regimens have shown improved responses and are considered important for current chemotherapy. However, intrinsic resistance is one of the causes of limited responsiveness to 5-FU-based chemotherapy (5, 6).

The antitumor activity of 5-FU is mediated by its metabolites and involves the irreversible inhibition of thymidylate synthase (TS) by fluorodeoxyuridine monophosphate (FdUMP), incorporation of fluorouridine triphosphate (FUTP) into RNA, and incorporation of uracil and 5-FU into DNA (4, 5). It has been demonstrated that both TS inhibition and FUTP incorporation into RNA play substantial roles in 5-FU-mediated cytotoxicity (7-10). TS is a critical enzyme for DNA replication and cell growth because it is the only de novo source of deoxythymidine monophosphate, the thymine nucleotide precursor, for DNA synthesis, and its high expression level in tumors has been associated with resistance to 5-FU-based chemotherapy (5, 11). In contrast, the contribution of uracil and 5-FU incorporation into DNA to the efficacy of treatment has not been clarified yet, despite extensive examination of the mode of action (12, 13). TS inhibition results in the depletion of deoxythymidine triphosphate (dTTP) and massive accumulation of deoxyuridine monophosphate (dUMP) in cancer cells. The accumulated dUMP is subsequently phosphorylated to its triphosphate, dUTP. Not only for dUMP, 5-FU itself is also metabolized to various nucleotides, including FdUTP, via de novo nucleotide synthesis pathway. Under conditions of dTTP depletion by TS inhibition, dUTP and FdUTP are misincorporated into DNA by DNA polymerase. To prevent the incorporation of aberrant bases into DNA, deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase) immediately catabolizes dUTP and FdUTP into their monophosphate forms. Thus, misincorporation of 5-FU and uracil is
tightly restricted via an intrinsic pathway. This notion is supported by a substantial increase in sensitivity to TS inhibitors when dUTPase expression is suppressed (12, 13) and by a poor response to TS inhibitors in cells highly expressing dUTPase (3, 14-16). On the other hand, once 5-FU and uracil are misincorporated into DNA, they are immediately excised from DNA by uracil-N-glycosylase (UNG) as a backup mechanism. Nevertheless, the expression levels of UNG showed little effect on sensitivity to TS inhibitors (17, 18); therefore, it has been suggested that dUTPase plays a primary role in the mechanism against misincorporation of 5-FU and uracil. A significant increase in dUTPase expression is observed in various cancers, and it has been suggested that higher expression leads to resistance to 5-FU-based chemotherapy (13, 16, 19-22). The critical role of dUTPase in 5-FU and uracil misincorporation-mediated cytotoxicity makes it a potential target for cancer treatments by combining a dUTPase inhibitor with a TS inhibitor (23-25).

In addition to intracellular 5-FU metabolism, it is important to consider 5-FU catabolism in the liver for further improvement of 5-FU-based chemotherapy (26, 27). Capecitabine is a stepwise activated prodrug of 5-FU designed to be selectively released in a tumor (28), and it is used as a platform for the treatment of advanced colorectal and breast cancers. Although there is substantial evidence supporting the use of capecitabine for these indications, patients experience several adverse effects such as GI toxicity, hematological toxicity, and hand-foot syndrome (HFS) (29). HFS is not a life-threatening toxicity, but it is frequently observed on treatment with capecitabine and seriously affects the patient’s quality of life; this results in dose reduction or changing of treatment schedule. The precise mechanism of HFS occurrence is not been fully understood; however, the catabolizing pathway of 5-FU could partly contribute because a retrospective analysis indicated that inhibitors of dihydropyrimidine dehydrogenase (DPD), a rate-limiting enzyme of 5-FU degradation in the liver, diminish the incidence of HFS upon continuous treatment with 5-FU (27).

Based on the above information, we attempted to develop an orally available dual inhibitor of dUTPase and DPD to propose a novel strategy for advanced oral fluoropyrimidine-based chemotherapy (Fig. 1). In this study, we describe the design concept of TAS-114, a novel potent small
molecule inhibitor of dUTPase and DPD under phase 1 and phase 2 combination trials, and evaluated its preclinical potential to improve the therapeutic efficacy of fluoropyrimidines.
Materials and Methods

Chemical compounds

TAS-114 \(\{N-[(1R)-1-[3-(cyclopentyloxy)phenyl]-ethyl]-3-[(3,4-dihydro-2,4-dioxo-1(2H)-pyrimidinyl)methoxy]-1-propanesulfonamide\}\) (Fig. 2A, method for the synthesis is given in the Supplementary Fig. S1), tegafur, gimeracil, and potassium oteracil were synthesized at Taiho Pharmaceutical Co., Ltd. S-1 is a combination form of tegafur/gimeracil/potassium oteracil at the molar ratio of 1:0.4:1 (see ref. 26 for chemical structures of these three components). 5-FU, 2′-deoxy-5-fluorouridine (FdUrd), and paclitaxel were obtained from Wako Pure Chemical Industries, Ltd. Capecitabine was obtained from F. Hoffmann-La Roche, Ltd. \([6-\text{^3}H]\)-FdUMP (666 GBq/μmol), [methyl-\text{^3}H]-TTP (2.52 TBq/μmol), [5-\text{^3}H]-UTP (652 GBq/μmol), [5-\text{^3}H]-dCTP (988 GBq/μmol), and [5-\text{^3}H]-dUTP (592 GBq/μmol) were obtained from Moravek Biochemicals, Inc.

Cell lines and a tumor xenograft

The HeLa and NUGC-4 cell lines were obtained from the Health Science Research Resources Bank. The NCI-H441, HT-29, and CFPAC-1 cell lines were obtained from the American Type Culture Collection. The MCF-7 cell line was obtained from DS Pharma Biomedical Co., Ltd. All cell lines were reauthenticated in 2012 by short tandem repeat-based DNA profiling. The MX-1 xenograft was acquired from Central Institute for Experimental Animals and reauthenticated in 2013 by short tandem repeat-based DNA profiling.

Cloning, expression, and purification of recombinant human dUTPase

The cDNA of human dUTPase was subcloned into the expression vector pET19b. The construct was then transformed into *Escherichia coli* BL21(DE3) cells (Novagen), and the cells were incubated in Luria broth at 37°C. Protein expression was induced with 0.01 mmol/L isopropyl-\(\beta\)-D-thiogalactopyranoside at an optical density of 0.6 at 595 nm. The cell pellet was resuspended in an ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl,
and 1 mmol/L dithiothreitol. After sonication, the debris was removed by centrifugation (10,000 × g, 30 min). The supernatant was applied to a nickel-nitrilotriacetic acid affinity gel, and the 6XHis-tag was removed by digestion with enterokinase in the lysis buffer for 12 h. The protein solutions used for crystallization were gel-filtered in the lysis buffer on a preparative grade Superdex 75 column (GE Healthcare UK Ltd).

**Co-crystal structure analysis**

Crystals of dUTPase complexed with TAS-114 were grown using the hanging-drop vapor diffusion method from a reservoir solution containing 15-20% polyethylene glycol 4000, 150 mmol/L CH$_3$COONa·3H$_2$O, 15% (v/v) glycerol, 5 mmol/L Zn(CH$_3$COO)$_2$·2H$_2$O, and 50 mmol/L Tris-HCl, pH 7.0, at 25°C. The protein was concentrated to 20 mg/mL, and the compound was added to a final concentration of 0.1 mmol/L. For data collection, the crystals were transferred to drops containing the equivalent mother liquor with 25% glycerol. Diffraction data were collected at the KEK Photon Factory Beamline BL-17A using a charge-coupled device area detector (ADSC Quantum 270). The data were processed using the CrystalClear software package (Rigaku Corporation). The space group was P2$_1$3. The structure of dUTPase complexed with the inhibitor was solved by molecular replacement using the program MOLREP (30) from the CCP4 suite (31). The search model was based on the human dUTPase structure [Protein Data Bank (PDB) ID: 1Q5U]. The structure of dUTPase complexed with the inhibitor was refined using REFMAC5 (32). Manual rebuilding of the models and interpretation of the electron density map were performed using COOT (33). The final model had the following R-values: $R_{\text{work}} = 17.0\%$ and $R_{\text{free}} = 17.3\%$. The summary of statistics from data collection and refinement is provided in Supplementary Table S1.

**Evaluation of TAS-114 enzyme selectivity**

HeLa cell pellets were mixed with an extraction solution (three volumes per cell pellet) containing 10 mmol/L Tris-HCl (pH 7.5), 4 mmol/L MgCl$_2$, 2 mmol/L 2-mercaptoethanol, 0.2 mmol/L...
phenylmethylsulfonyl fluoride, and 10% glycerol and sonicated. The supernatant collected after centrifugation was used as the cell extract.

The substrates ([methyl-\(^{3}\)H]-TTP, [\(^{5}\)H]-UTP, [\(^{5}\)H]-dCTP, and [\(^{5}\)H]-dUTP) mixed at 1 \(\mu\)mol/L with a diluted cell extract (1:400) were incubated in a reaction buffer containing 50 mmol/L Tris-HCl (pH 7.4), 4 mmol/L MgCl\(_2\), 2 mmol/L 2-mercaptoethanol, and 0.1% bovine serum albumin for 30 min at 37\(^{\circ}\)C in the absence or presence of TAS-114.

The amounts of the substrates were quantified by radio-high-performance liquid chromatography (HPLC). The inhibitory activity of TAS-114 was calculated from its inhibition rate of substrate hydrolysis.

**Quantification of cell proliferation**

Cellular proliferation was evaluated by crystal violet staining based on the method of Saotome et al. (34). Cancer cells were plated in a 96-well plate and cultured for 24 h. Then, drugs were added and incubated for 72 h. Cells were fixed with 25% glutaraldehyde and stained with a 0.05% crystal violet solution in 20% methanol. An extraction solution (0.1 mol/L NaH\(_2\)PO\(_4\) and 100% ethanol, 1:1) was added, and the absorbance at 540 nm was measured.

**Pharmacokinetic and pharmacodynamic studies**

For pharmacokinetic (PK) and pharmacodynamic (PD) studies of TAS-114 in combination with capecitabine, the test compounds were orally administered to BALB/c nude mice (Clea Japan, Inc) bearing MX-1 human breast cancer xenografts because rats could not be used due to lack of one of the enzymes converting capecitabine to 5-FU (35). Plasma and tumor samples were collected from the mice (n = 3 at each sampling point per group). The concentrations of 5-FU and TAS-114 in plasma samples were quantified by liquid chromatography-tandem mass spectrometry, and the area under the concentration-time curve (\(AUC_{0-6}\)) was calculated. To evaluate TS and dUTPase inhibition, the levels of free FdUMP, dUMP, and free TS were measured in the tumor, and the \(AUC_{last}\) values of intratumoral...
free FdUMP and dUMP were calculated.

For the PD study of TAS-114 in combination with S-1, the test compounds were orally administered to F344 nude rats bearing MX-1 (n = 5) because the PK profiles of the components of S-1 were comparable with those of humans. Tumor samples were collected 4 h after administration, and the levels of free FdUMP, dUMP, and free TS were measured in the tumor.

**Measurement of free FdUMP and dUMP levels in tissues**

Perchloric acid (0.48 N, five volumes of tissue weight) was added to tissue samples, followed by homogenization. A sufficient amount (two or three volumes) of a dichloromethane solution containing 0.5 N tri-\(n\)-octylamine was added to the supernatant obtained by centrifugation (800 × g, 3 min, 4°C) and mixed by vortexing. The aqueous layer was collected after centrifugation and analyzed as an acid-soluble fraction. The measurement of free FdUMP levels was conducted using a partially modified FdUMP-TS binding assay developed by Takeda et al. (36). FdUMP, an active metabolite of 5-FU, irreversibly binds to TS in the presence of 5,10-methylene tetrahydrofolate acid. Based on this property, free FdUMP present in the acid-soluble fractions prepared from tissues was competitively bound to human recombinant TS in the presence of [6-\(^3\)H]-FdUMP. The amount of bound [6-\(^3\)H]-FdUMP was measured using a liquid scintillation counter (Perkin Elmer A290001). The amount of dUMP in the acid-soluble fractions was quantified by HPLC. The HPLC analyses were performed using a Shimadzu LC-VP Series HPLC system with a UV detector set to 265 nm. Chromatographic separations were performed on an Atlantis dC18 column (4.6×250 mm, 5 μm) (Waters Corporation) kept at 25°C using a flow rate of 0.8 mL/min. Mobile phase A was a mixture of buffer A and buffer B (60: 40 v/v) and mobile phase B used buffer B. Buffer A contained 10 mmol/L KH\(_2\)PO\(_4\), 10 mmol/L tetrabutylammonium-hydroxide (TBA-OH), and 0.25 vol% methanol (pH 6.7). Buffer B contained 50 mmol/L KH\(_2\)PO\(_4\), 5.6 mmol/L TBA-OH, and 30 vol% methanol. The gradient elution time program and final % concentrations of buffer B were, respectively, 0-60 min 40-100%, 60-80 min 100%, 80-81 min 100-40%, and 81-95 min 40%.
Measurement of free TS levels in tissues

A homogenization buffer containing 200 mmol/L Tris-HCl (pH 7.4), 15 mmol/L 5′-CMP, 100 mmol/L NaF, and 20 mmol/L 2-mercaptoethanol was added to tissue samples. The samples were homogenized and then centrifuged at 105,000 × g for 60 min at 4°C. The supernatant was collected, and free TS levels were measured using a partially modified FdUMP-TS binding assay developed by Takeda et al. (36). Excess amounts of [6-3H]-FdUMP and 5,10-methylene tetrahydrofolic acid were added to the samples. Free TS bound to [6-3H]-FdUMP with 5,10-methylene tetrahydrofolic acid to form a ternary complex. The amounts of complex formed were measured using a liquid scintillation counter (Perkin Elmer A290001). The concentration of free TS was calculated and normalized to the protein concentration.

In vivo efficacy studies

MX-1 fragments were implanted subcutaneously into the right chest of each F344 nude rat (Clea Japan, Inc.) or BALB/c nude mouse (Clea Japan, Inc.). The tumor volume (TV, mm³) was calculated as the [length (mm) × width (mm)²]/2. Five animals were assigned to each group such that the mean TV was equal (approximately 200 mm³ in the mouse model and 500 mm³ in the rat model) among the groups for each experiment at day 0. Test compounds were orally administered every day from day 1. The relative TV (RTV) on day 15 was calculated as the ratio of TV on day 15 to that on day 0. The inhibition rate of tumor growth (%) on day 15 was calculated based on RTV as 100 × [1 − (mean RTV of drug-treated group)/(mean RTV of control group)]. The body weight (BW) change (%) during the dosing period was calculated as 100 × [(BW on each measurement day) − BW_initial]/BW_initial, where BW_initial refers to BW on day 0. Capecitabine was administered at doses of 71 to 809 mg/kg/day, alone or in combination with TAS-114 at 37.5 to 1,200 mg/kg/day, and TAS-114 alone was administered at 1,200 mg/kg/day to nude mice bearing MX-1. Gimeracil at 0.7 mg/kg/day was co-administered with capecitabine at 240 mg/kg/day. S-1 was administered at 15 mg/kg/day, alone or in combination with...
TAS-114 at 75 or 300 mg/kg/day, to nude rats bearing MX-1. Dunnett’s test was used as a statistical method to compare the tumor volume data between the drug-treated groups and the control group. Either Dunnett’s or the Welch t-test was used to compare the tumor volume data between the groups treated with a combination of TAS-114 and the groups treated with capecitabine or S-1 alone. $P < 0.05$ was considered statistically significant. The rats and mice were euthanized when they became moribund. All animal experiments were conducted in accordance with the guidelines for animal experiments of Taiho Pharmaceutical Co., Ltd and were reviewed and approved according to regional Institutional Animal Care and Use Committees.
Results

Design concept of dual dUTPase/DPD inhibitor

It is already known that N-1 substituted uracil derivatives such as N1-benzyluracil show inhibitory activities against mouse dihydrouracil dehydrogenase (DHUDase, EC 1.3.1.2) (37). Additionally, we previously demonstrated that they also show potent inhibitory activities against human dUTPase (38-41). Therefore, we anticipated that our N-1 substituted uracil derivatives would potentially exhibit dual inhibitory activities against human dUTPase and DPD.

To confirm TAS-114 as a clinical candidate, we considered dUTPase inhibitory activity to be more important because no other dUTPase inhibitor has been evaluated in clinical studies and even a moderate inhibitory activity against DPD could be sufficient to reduce the incidence of HFS (27).

TAS-114 is a potent inhibitor of dUTPase, showing moderate inhibition of DPD

We proposed the inhibition mode of TAS-114 against dUTPase based on their co-crystal structure. Unlike the computational docking results reported by Hagenkort et al. (42), the terminal phenyl ring of TAS-114 occupied a hydrophobic region formed by Val65, Ala90, Ala98, and Val112, instead of the phenyl ring of Phe158 of dUTPase, and was stacked with its uracil ring. This stabilization of TAS-114 in the catalytic pocket may be responsible for its inhibitory activity. Moreover, it was revealed that the m-cyclopentyloxy moiety of the TAS-114 terminal phenyl ring occupied another hydrophobic space, formed by Val66, Lys67, Asn108, Val109, and Gly110, and this hydrophobic interaction might potentiate its inhibitory activity (Fig. 2B).

Kinetic analysis of dUTPase inhibition revealed that TAS-114 competitively inhibited dUTPase in the dUTP binding step (Supplementary Fig. S2B and C). The $K_i$ value of TAS-114 was calculated to be 0.13 $\mu$mol/L, which was significantly lower than the $K_m$ value (1.3 $\mu$mol/L) of dUTP under the same condition (Supplementary Fig. S2A and B). The substrate analysis of FdUTP revealed that TAS-114 exhibited enzyme inhibition values similar to those of kinetic analysis ($K_i$ value: 0.10 $\mu$mol/L, $K_m$ value: 1.5 $\mu$mol/L, Supplementary Fig. S2D). Together with the results of co-crystal structure analysis,
it was observed that TAS-114 competitively inhibited dUTPase at the substrate-binding pocket (Fig. 2B).

To investigate the selectivity of TAS-114 for dUTPase, we measured the inhibitory activity of TAS-114 on dUTP dephosphorylation in HeLa cell extracts. TAS-114 inhibited dUTP degradation in a crude cell extract; however, it did not affect the degradation of other nucleoside triphosphates (NTPs) in the extract. More specifically, dUMP was the predominant product of dUTP degradation in the extract, which was undoubtedly due to the strong activity of dUTPase. Nevertheless, TAS-114 changed the dUTP degradation profile to almost the same pattern as that of other NTPs (Fig. 2C). Thus, TAS-114 selectively inhibited the hydrolysis of dUTP to dUMP in cancer cells.

In addition to dUTPase inhibition, TAS-114 showed DPD inhibitory activity as expected. We analyzed the mode of DPD inhibition by TAS-114 using a human liver S9 microsomal fraction and the cytosol. TAS-114 inhibited DPD in a concentration-dependent manner, and its inhibitory activity was lower than that of gimeracil, but approximately eight-fold higher than that of uracil, an intrinsic substrate of DPD (Supplementary Fig. S3A). In the evaluation of the time-dependent inhibitory effect, bromovinyluracil, an irreversible DPD inhibitor, inhibited DPD at a lower concentration after preincubation. In contrast, the inhibitory activity of TAS-114 did not change after preincubation, similar to uracil, a reversible inhibitor of DPD (Supplementary Fig. S3B). Therefore, TAS-114 acted as a reversible inhibitor of DPD.

**TAS-114 significantly enhances cytotoxicity of fluoropyrimidines**

Next, we evaluated the ability of TAS-114 to enhance cytotoxicity of fluoropyrimidines in HeLa cells. TAS-114 showed little intrinsic activity at 10 μmol/L (Supplementary Table S2); however, it clearly increased the cytotoxicity of FdUrd against various cancer cell lines (Fig. 3A). TAS-114 also increased the cytotoxicity of 5-FU, although the increase was relatively smaller than that of FdUrd (Supplementary Table S2). In contrast to the significant increase of fluoropyrimidine activities, TAS-114 showed little effect on the cytotoxicity of paclitaxel, which has a different mode of action.
Because TAS-114 is a dual dUTPase/DPD inhibitor, we evaluated the contributions of both the enzymes to the increase of 5-FU cytotoxicity in HeLa cells expressing DPD. Compared with the significant increase of cytotoxicity in combination with TAS-114, the potent DPD inhibitor gimeracil showed little potentiation of 5-FU cytotoxicity in HeLa cells (Fig. 3B). It is considered that the inhibition of dUTPase rather than DPD plays a major role in the enhancement of 5-FU cytotoxicity by TAS-114 in HeLa cells.

TAS-114 increased the plasma level of 5-FU derived from capecitabine through DPD inhibition and suppressed dUTPase enzymatic product levels in tumors through dUTPase inhibition

DPD is a rate-limiting enzyme for 5-FU catabolism in the liver (43), and its inhibition increases 5-FU bioavailability; therefore, a DPD inhibitor can reduce the pharmacologically required dose of 5-FU (26). Capecitabine is a multistep activated oral prodrug releasing 5-FU. DPD is the primary enzyme responsible for catabolism of 5-FU from capecitabine (44). Reduction of the total dose of capecitabine by inhibiting DPD might be beneficial in terms of incidence of adverse effects such as HFS (Fig. 1). Therefore, we evaluated the potency of TAS-114 in DPD inhibition upon oral administration of TAS-114 together with capecitabine.

As expected, the plasma 5-FU exposure level was elevated in a TAS-114 dose-dependent manner (Table 1). Its activity was moderate compared with that of the potent DPD inhibitor gimeracil, as shown in an in vitro study (Supplementary Fig. S3).

Then, we conducted a detailed analysis of PD parameters in a human breast cancer-derived MX-1 xenograft mouse model because MX-1 highly expresses dUTPase (Supplementary Fig. S4A). Capecitabine alone increased TS inhibition in a dose-dependent manner, and the intratumoral free FdUMP level increased along with the increase in TS inhibition. As expected, the combination with gimeracil showed similar effects by increasing exposure to 5-FU through strong DPD inhibition. In contrast, TAS-114 dramatically decreased the accumulation of free FdUMP and dUMP within the tumor in a dose-dependent manner despite the increase in 5-FU exposure, and did not significantly
influence TS inhibition (Table 1). These results indicate that TAS-114 modulates the 5-FU and uracil misincorporation pathway by inhibiting dUTPase in the tumor. TAS-114 exerts its dual inhibitory effects on dUTPase and DPD when orally administered with capecitabine.

**Addition of TAS-114 resulted in greater antitumor activity at a reduced dose of capecitabine**

Next, we conducted a titration study of capecitabine, TAS-114, and their combinations to prove the dual inhibitor concept based on efficacy and toxicity (Fig. 4A). The tolerable doses of capecitabine decreased in a TAS-114 dose-dependent manner; therefore, co-administration of TAS-114 at 600 mg/kg reduced the pharmacologically required dose of capecitabine to approximately one-third of its toxic dose in monotherapy. The antitumor activities of various combinations exceeded the maximum potency of capecitabine monotherapy, even at lower doses of the prodrug.

In contrast, although the potent DPD inhibitor gimeracil allowed a reduction of capecitabine dose similar to TAS-114, it did not show therapeutic improvement at a dose that appeared to be equivalent to the DPD-inhibiting dose based on toxicity (Fig. 4B).

**dUTPase inhibition plays a crucial role in tumor-selective enhancement of 5-FU-mediated antiproliferative activity**

Gimeracil dramatically increased the bioavailability of 5-FU but could not therapeutically improve the antitumor activity of capecitabine. This suggests that systemic inhibition of DPD has no or little influence on the therapeutic window. Because a greater activity was achieved in combination with TAS-114, dUTPase inhibition is considered the primary reason for the enhanced antitumor efficacy of capecitabine.

S-1 is a fixed-combination drug of tegafur (prodrug of 5-FU), gimeracil (DPD inhibitor), and oteracil (orotate phosphoribosyltransferase inhibitor), which has been widely used in Asian countries as an oral fluoropyrimidine drug (26, 45). To confirm that dUTPase inhibition contributes to the synergistic potentiation of antitumor activity, we conducted a combination study of TAS-114 with S-1
to minimize the DPD inhibitory activity of TAS-114 in rats, which are considered to have 5-FU PK properties after administration of S-1 similar to those in humans.

TAS-114 clearly enhanced the antitumor activity of S-1 in an MX-1 xenograft rat model without increasing BW loss (Fig. 5A and B). It showed similar results in various cancer xenograft models in combination with S-1 and prolonged the mouse lifespan in a survival model (Supplementary Fig. S4; Supplementary Table S3). In the PK analysis of the combination of S-1 and TAS-114, co-administration of TAS-114 did not affect exposure to tegafur or 5-FU (Supplementary Fig. S5A). Furthermore, exposure to TAS-114 was not different between TAS-114 administration alone or in combination with S-1 (Supplementary Fig. S5B). This result indicates that, as expected, the DPD inhibitory activity of TAS-114 is completely masked by gimeracil when TAS-114 is orally administered with S-1. In contrast, the intratumoral concentrations of the dUTPase products dUMP and free FdUMP were dramatically decreased upon combination with TAS-114, whereas the strong inhibition of TS was retained (Fig. 5C). These observations demonstrate that dUTPase inhibition of TAS-114 leads to the enhancement of antitumor efficacy of S-1. Furthermore, after administration of S-1 to rats, these dUTPase products highly and selectively accumulated in the tumor compared to normal tissues; however, TS was inhibited in both tumor and normal tissues (Fig. 5D). These differences indicate that TAS-114 in combination with S-1 leads to tumor-selective modulation and appear to reflect cancer vulnerability when a dUTPase inhibitor is used with a 5-FU-based drug.
Discussion

In this study, we aimed to develop a novel dUTPase/DPD inhibitor and found that it significantly improved the therapeutic efficacy of fluoropyrimidines.

dUTPase selectively catalyzes dUTP and FdUTP hydrolysis and is a gatekeeper protein, which prevents the incorporation of aberrant bases, such as uracil and 5-FU, into DNA. Over the last few decades, various studies have been conducted on the function of dUTPase in cancer cells, and it has been demonstrated that dUTPase contributes to poor sensitivity to 5-FU drugs (12-16). Based on these data, dUTPase is considered a potential therapeutic target for the treatment of cancer. We developed a dUTPase inhibitor to maximize the antitumor activity of 5-FU drugs.

TAS-114 is a novel, orally available dual dUTPase/DPD inhibitor that shows a potent dUTPase inhibitory activity without any effect on the catabolism of other nucleotides and a modest activity effect on DPD inhibition. In this study, we demonstrated that a dual dUTPase/DPD inhibitor has great potential to be used in advanced 5-FU-based chemotherapy.

TAS-114 itself shows little intrinsic inhibitory activity on the growth of cancer cells; however, it remarkably enhances the cytotoxicity of fluoropyrimidines. The increase of FdUrd cytotoxicity in combination with TAS-114 is more significant than that of 5-FU. FdUrd is converted into FdUMP in one step and the primary target of FdUrd cytotoxicity is TS inhibition. The modulation by TAS-114 is based on TS inhibition; therefore, it is thought to be easier to detect the enhancement effect induced by TAS-114 in combination with FdUrd.

The enhancement of 5-FU cytotoxicity in combination with TAS-114 was not influenced in the presence of the potent selective DPD inhibitor gimeracil in HeLa cells expressing DPD. The difference in 5-FU cytotoxicity upon DPD inhibition was small, and similar results were reported even in cells expressing high levels of DPD (46). These results indicate that dUTPase inhibition is the primary mechanism of TAS-114-mediated enhanced cytotoxicity.

In combination with capecitabine, TAS-114 acts as a dual inhibitor of dUTPase and DPD. Oral administration of TAS-114 with capecitabine elevated the plasma 5-FU level by inhibiting DPD. In
contrast, intratumoral levels of the dUTPase products FdUMP and dUMP decreased drastically. TS inhibition by TAS-114 combination was the same as that by capecitabine alone, indicating that TAS-114 inhibited dUTPase in the tumor in vivo. DPD inhibition by TAS-114 reduced the required dose of capecitabine to approximately 30% of the dose of capecitabine monotherapy. Even though TAS-114 reduced the required dose of capecitabine, greater antitumor activities were achieved at various doses. In contrast, the selective DPD inhibitor gimeracil was not able to improve the therapeutic window, although it reduced the required dose of capecitabine, similar to TAS-114.

It is believed that the combined use of DPD inhibitors reduces the incidence of HFS caused by capecitabine (27). A retrospective analysis suggested that the incidence of HFS could be suppressed even in combination with weak DPD inhibitors, which are expected to reduce the dose of fluoropyrimidines in half compared to monotherapy (27). A PK/PD study of single-agent TAS-114 in healthy volunteers showed that it inhibited DPD at a higher dose (47). This observation suggests that TAS-114 works as a dual inhibitor in humans when used at a higher dose level, and improvement of therapeutic efficacy is expected to be demonstrated in clinical combination study.

Capecitabine is a multistep activated prodrug of 5-FU, and thymidine phosphorylase (TP) is considered to be one of the enzymes involved in its conversion to 5-FU in tumors (28). TAS-114 does not inhibit the pathway of 5-FU activation by TP, and it does not affect the antitumor activity of capecitabine in TP-overexpressing tumor xenografts, which are susceptible to capecitabine (48). TAS-114 can modulate the antitumor activity of capecitabine without diminishing its intrinsic activity.

TAS-114 also enhanced the antitumor activity of S-1, which contains a potent DPD inhibitor as one of the components, in various xenograft models. DPD inhibition by TAS-114 is completely minimized upon co-administration of gimeracil, because the plasma level of 5-FU was equivalent to that obtained with S-1 alone. TAS-114 reduces the accumulation of dUTPase enzymatic products in the tumor under the same conditions of treatment. These data clearly demonstrate that dUTPase inhibition by TAS-114 is responsible for the enhancement of antitumor activity.

In addition, TAS-114 enhanced the antitumor activity of S-1 without increasing BW loss. After
administration of S-1, the intratumoral levels of the dUTPase products dUMP and free FdUMP were higher than the levels in normal tissues. In contrast, TS inhibition was observed in various normal tissues after S-1 monotherapy. These data indicate that TAS-114 in combination with S-1 leads to tumor-selective modulation and results in enhancement of efficacy without additional toxicity. Similar to S-1, administration of capecitabine to mice resulted in selective accumulation of dUTPase products in tumors (49). A high level of free FdUMP has been observed in clinical tumors (50); thus, dUTPase inhibition is expected to enhance the tumor-selective efficacy of 5-FU-based chemotherapy in the clinical setting compared to that observed upon co-administration of leucovorin, which modulates the TS inhibitory activity of 5-FU.

In summary, TAS-114 is a potent dUTPase inhibitor with modest inhibitory activity against DPD. Oral co-administration of TAS-114 with 5-FU-based drugs dramatically improved their antitumor activities. Dual dUTPase/DPD inhibition is a novel strategy for further improvement of 5-FU-based chemotherapy. The concomitant use of the dUTPase/DPD dual inhibitor is expected to provide a novel strategy for advanced fluoropyrimidine-based chemotherapy, and the therapeutic activity of TAS-114 should be evaluated in clinical settings.
Acknowledgments

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References


Table 1. Pharmacokinetic and pharmacodynamic studies of capecitabine in combination with TAS-114 or gimeracil.
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<td>Free TS (pmol/mg protein)</td>
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NOTE: Plasma and tumor tissues were collected at 0.5, 1, 2, 4, and 6 h after drug administration from three nude mice with MX-1 (human breast cancer) at each sampling point per group.
Figure legends

Figure 1.
Concept of dual inhibition with combination treatment of capecitabine and TAS-114. Combination with TAS-114 can reduce the maximum tolerated dose of capecitabine by DPD inhibition, and it significantly enhances the antitumor efficacy through dUTPase inhibition. TAS-114 improves the therapeutic window of capecitabine.

Figure 2.
TAS-114, a novel dual dUTPase/DPD inhibitor, and its enzyme inhibition kinetics and selectivity in nucleotide hydrolysis. (A) Chemical structure of TAS-114. (B) X-ray co-crystal structure of TAS-114 with human dUTPase (PDB code: 5H4J). (C) Effects of TAS-114 on hydrolysis of nucleotide triphosphates in a HeLa cell extract. Substrates (TTP, UTP, dCTP, and dUTP) were mixed at 1 μmol/L with a HeLa cell extract and incubated for 30 min at 37°C. The inhibitory activity of TAS-114 at 10 μmol/L was determined as the percent decrease in substrates following treatment with the cell extract. Triphosphate (TP), diphosphate (DP), monophosphate (MP), and nucleoside (Ns).

Figure 3.
TAS-114 enhances the cytotoxicity of a TS inhibitor via dUTPase inhibition. (A) TAS-114 enhanced FdUrd, but not paclitaxel-induced cytotoxicity in various cancer cell lines. Cell growth inhibition was determined by crystal violet staining. The cells were treated with FdUrd and paclitaxel in combination with TAS-114 at 10 μmol/L for 72 h. (B) Comparison of the effects of a dual dUTPase/DPD inhibitor (TAS-114) and a potent DPD inhibitor (gimeracil) on HeLa cells. The cells were treated with 5-FU, TAS-114 at 10 μmol/L, and gimeracil at 10 μmol/L for 72 h, and cell growth inhibition was determined by crystal violet staining.

Figure 4.
Efficacy and toxicity of capecitabine and TAS-114 combination. (A) Efficacy and toxicity titration study of capecitabine and TAS-114 combination. Dose responses to combined administration of TAS-114 with capecitabine in nude mice bearing MX-1 tumors. The mice (n = 5 per group) were orally administered capecitabine and/or TAS-114 at various doses from day 1 to day 14. Each value in the cell is the tumor growth inhibition rate (%) on day 15. *P < 0.05 and **P < 0.01 versus control. #P < 0.05 and ##P < 0.01 versus capecitabine, 539 mg/kg/day (maximum tolerated dose of capecitabine alone). (B) Antitumor efficacy and toxicity of capecitabine plus a dual inhibitor of dUTPase and DPD (TAS-114) versus a selective DPD inhibitor (gimeracil) in an MX-1 xenograft mouse model. Nude mice bearing MX-1 tumors (n = 5 per group) were orally administered capecitabine at 240, 539, and 809 mg/kg/day, TAS-114 at 600 mg/kg/day, and gimeracil at 0.7 mg/kg/day from day 1 to day 14. Data are presented as the mean ± standard deviation (SD). **P < 0.01 versus control. #P < 0.05 and ##P < 0.01 versus capecitabine (539 mg/kg/day).

Figure 5.

Antitumor efficacy of the S-1 and TAS-114 combination in a xenograft model. Antitumor efficacy (A) and toxicity (B) of S-1 in combination with TAS-114 in an MX-1 xenograft rat model. Nude rats bearing MX-1 tumors (n = 5 per group) were orally administered S-1 at 15 mg/kg/day and TAS-114 at 75, 300, and 1,000 mg/kg/day from day 1 to day 14. Data are presented as the mean ± SD. **P < 0.01 versus control. ##P < 0.01 versus S-1 alone. (B) Body weight change in the rats corresponding to (A). (C) Pharmacodynamic study in rats. Nude rats bearing MX-1 tumors (n = 5 per group) were orally administered S-1 at 15 mg/kg and TAS-114 at 75, 300, and 1,000 mg/kg. Tumor samples were collected 4 h after oral administration. Data are presented as the mean ± SD. (D) dUMP, FdUMP, and free TS in tumor and normal tissues after administration of S-1 to rats. S-1 was orally administered at a dose of 18 mg/kg to nude rats bearing MX-1 tumors (n = 3 per group). Tumor and normal tissue samples were collected from non-treated and treated rats 2 h after oral administration. Data are presented as the mean ± SD.
Figure 1

The diagram illustrates the metabolic pathway of 5-fluorouracil (5-FU) and its interaction with other molecules such as thymidylate synthase (TS), deoxycytidine monophosphate deaminase (DPD), and other enzymes involved in folate metabolism.

- **Capcitabine** leads to **5-FU** production.
- **5-FU** is further metabolized to **FdUrd** and **FdUMP**.
- **FdUMP** is converted to **dUMP** by **dUTPase**.
- **TAS-114** inhibits **DPD** and **TAS-114**.
- **Adverse effect** includes **FBAL**.
- The **Liver** metabolizes **5-FU**.
- **Dose reduction** impacts the overall efficacy.

The diagram also highlights the importance of **TP** in the metabolic pathway and the role of **OPRT**, **RNR**, and **TK** enzymes in the conversion of **FUMP**, **FUDP**, and **FUTP** to **FdUTP**.
Figure 2

(A) A novel dUTPase/DPD dual inhibitor TAS-114

Ki for dUTPase = 0.13 μmol/L (substrate: dUTP)

Ki for DPD = 2.14 μmol/L (substrate: 5-FU)

(B)

(C)

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Figure 3

(A)

(B)
**Figure 4**

**(A)**

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<td>-2</td>
<td>18</td>
<td>51 **</td>
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**Efficacy**

The number in each column is inhibition ratio (%) of tumor growth at day 15

- **Green**: Therapeutically improved dose
- **Light gray**: Equivalent to Capecitabine monotherapy (± 10% difference to Capecitabine 539 mg/kg)

**Toxicity**

- **Orange**: Intolerable dose
- **Red**: 20% of Body weight loss at day 15
- **Red**: Maximum tolerated dose (MTD)
- **Not examined**

**(B)**

**Efficacy**

- Control
- Capecitabine (240 mg/kg)
- Capecitabine (539 mg/kg)
- Capecitabine (809 mg/kg)
- Capecitabine/TAS-114 (240/600 mg/kg)
- Capecitabine/gimeracil (240/0.7 mg/kg)

**Toxicity**

- Control
- Capecitabine (240 mg/kg)
- Capecitabine (539 mg/kg)
- Capecitabine (809 mg/kg)
- Capecitabine/TAS-114 (240/600 mg/kg)
- Capecitabine/gimeracil (240/0.7 mg/kg)
Molecular Cancer Therapeutics

TAS-114, a First-in-Class Dual dUTPase/DPD Inhibitor, Demonstrates Potential to Improve Therapeutic Efficacy of Fluoropyrimidine-based Chemotherapy

Wakako Yano, Tatsushi Yokogawa, Takeshi Wakasa, et al.

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