

# The mechanism underlying resistance to 5-fluorouracil and its reversal by the inhibition of thymidine phosphorylase in breast cancer cells

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**Abstract.** Fluoro-deoxyuridine monophosphate (FdUMP) is an active metabolite of 5-fluorouracil (5-FU) synthesized through two hypothesized pathways: The orotate phosphoribosyl transferase-ribonucleotide reductase (OPRT-RR) pathway and the thymidine phosphorylase-thymidine kinase (TP-TK) pathway. In the present study, the mechanism underlying 5-FU resistance was investigated, focusing on changes in 5-FU metabolism using MCF-7, 5-FU-resistant MCF-7/5-FUR, MDA-MB-231 and 5-FU-resistant MDA-MB-231/5-FUR breast cancer cells. The amount of FdUMP present following treatment with 5-FU was determined by the density of the upper band of thymidylate synthase detected by western blotting, and its changes were investigated. MCF-7/5-FUR cells exhibited 5-FU resistance (36.6-fold), and showed decreased OPRT (-69.3%) and TK (-42.6%) levels. MDA-MB-231/5-FUR cells also exhibited 5-FU resistance (15.8-fold), and showed decreased TP (-79.0%) and increased TK (+184%) levels. MCF-7/5-FUR and MDA-MB-231/5-FUR cells both showed decreased synthesis of FdUMP by 91 and 86%, respectively. In MCF-7 and MCF-7/5-FUR cells, the synthesis of FdUMP was

decreased when 5-FU was combined with an RR inhibitor, indicating that FdUMP was synthesized through the OPRT-RR pathway. The synthesis of FdUMP was decreased when 5-FU was combined with a TP inhibitor in MDA-MB-231 cells and combined with an RR inhibitor in MDA-MB-231/5-FUR cells, indicating that the synthesis pathway of FdUMP was changed from the TP-TK pathway to the OPRT-RR pathway on acquiring resistance to 5-FU. Notably, the synthesis of FdUMP was increased and the resistance to 5-FU was reversed in MCF-7/5-FUR cells (half maximal inhibitory concentration ( $IC_{50}$ ): 219.9 to 0.093  $\mu$ M) and MDA-MB-231/5-FUR cells ( $IC_{50}$ : 157.3 to 31.0  $\mu$ M) when 5-FU was combined with a TP inhibitor. In conclusion, the metabolism of 5-FU and the mechanism underlying the resistance to 5-FU differed among cell lines, and inhibition of TP reversed resistance to 5-FU, thus suggesting that the combination of 5-FU and a TP inhibitor may be considered a promising cancer therapy.

## Introduction

Breast cancer is one of the most common diseases worldwide (1), so developing effective breast cancer therapies is important. While surgery is the mainstay of treatment for most breast cancers, there are limitations to the benefits that surgery can provide, especially in patients with advanced disease. Therefore, the principal developments in cancer therapy are expected to be provided by drug therapy.

5-Fluorouracil (5-FU) has been a key drug for many other cancers (2), and its importance in breast cancer treatment has also been increasing recently in both the adjuvant setting (3,4) and metastatic setting (5). There are three speculated mechanisms of action for 5-FU: incorporation into RNA (6), incorporation into DNA (7), and inhibition of DNA *de novo* synthesis by inhibiting thymidine synthase (TS) (8). Among these speculated mechanisms, the inhibition of TS has received the most focus because many chemotherapeutic drugs similarly inhibit TS (9,10), and some drugs enhancing the inhibition of TS have been developed (11,12).

Fluoro-deoxyuridine monophosphate (FdUMP) is the key molecule synthesized from 5-FU in cancer cells. It inhibits TS by forming a ternary complex composed of TS, FdUMP and 5,10-methylenetetrahydrofolate ( $CH_2$ THF) (2). This metabolism is associated with the enzymes involved in the synthesis of deoxythymidine monophosphate (dTMP), which is

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**Abbreviations:** 5-FU, 5-fluorouracil;  $CH_2$ THF, 5,10-methylenetetrahydrofolate; CI, confidence interval; dT, thymidine; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FdU, fluoro-deoxyuridine; FdUMP, fluoro-deoxyuridine monophosphate; FUMP, 5-fluorouridine monophosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase;  $IC_{50}$ , half maximal inhibitory concentration; MTT, 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide; NT, nucleotidase; OPRT, orotate phosphoribosyl transferase; PBS, phosphate-buffered saline; RR, ribonucleotide reductase; SE, standard error; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; UMP, uridine monophosphate

**Key words:** breast neoplasms, 5-FU, metabolism, drug resistance, thymidine phosphorylase

necessary for the synthesis of DNA. There are two speculated pathways for synthesizing FdUMP: i) 5-FU is converted to 5-fluorouridine monophosphate (FUMP) by orotate phosphoribosyltransferase (OPRT) and then converted to FdUMP by several enzymes, including ribonucleotide reductase (RR), in a process known as the 'OPRT-RR pathway' derived from the *de novo* pathway of dTMP synthesis; or ii) 5-FU is converted to fluoro-deoxyuridine (FdU) by thymidine phosphorylase (TP) and then converted to FdUMP by thymidine kinase (TK), in a process known as the 'TP-TK pathway' derived from the salvage pathway of dTMP synthesis. These mechanisms are illustrated in Fig. 1A and B.

Long-term treatment with 5-FU or other anti-cancer drugs causes drug resistance, and overcoming resistance to 5-FU is important for improving breast cancer treatment. However, the changes in the metabolisms caused by the acquisition of resistance to 5-FU in breast cancer cells has never been clearly described. Therefore, in the present study, we focused on the changes in the metabolism of 5-FU and the synthesis of dTMP to elucidate the mechanism underlying resistance to 5-FU using 5-FU-resistant breast cancer cell lines.

## Materials and methods

**Drugs.** 5-FU was kindly provided by Kyowa Hakko. Tipiracil (TP inhibitor), hydroxyurea (RR inhibitor) and raltitrexed (TS inhibitor) were purchased from Sigma-Aldrich.

**Cell lines and cell culture.** MCF-7 and MDA-MB-231 cells obtained from ATCC were cultured in Dulbecco's modified Eagle's medium (D5921-500ML; Sigma-Aldrich; Merck KGaA) with 5% fetal bovine serum (10270-106; Thermo Fisher Scientific), L-Glutamine (073-05391; FUJIFILM Wako Pure Chemical Corporation), and Non-essential amino acid (M7145-100ML; Sigma-Aldrich; Merck KGaA). MCF-7/5-FUR and MDA-MB-231/5-FUR cells are 5-FU-resistant cell lines established in our institute by continuously exposing MCF-7 or MDA-MB-231 cells to 5-FU over a few years. The initial concentration of 5-FU was 0.1  $\mu$ M, and it was increased 2-fold once cell growth was confirmed, up to 10  $\mu$ M at 37°C. These cells were maintained in medium containing 2  $\mu$ M of 5-FU and cultured in drug-free medium for at least 2 weeks before experiments to eliminate the effects of 5-FU. The cell lines were incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

**Western blot analyses and antibodies.** The cells were lysed in RIPA buffer (Sigma-Aldrich; Merck KGaA) for 15 min on ice. The protein concentration of the lysates was measured using a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc.). The cell lysates were boiled in sample buffer solution (FUJIFILM Wako Pure Chemical Corporation). Total cell protein extracts (7  $\mu$ g/lane) were separated by 10% SDS-PAGE using SuperSep™ ACE (FUJIFILM Wako Pure Chemical Corporation) and transferred onto polyvinyl difluoride (PVDF) membranes (EMD Millipore). The membranes were blocked with PVDF blocking reagent (Toyobo Co., Ltd.) for 1 h. The membranes were then incubated with primary antibodies, such as anti-OPRT antibody (kindly provided by Taiho Pharmaceutical Company

(<https://www.taiho.co.jp/en/>); 1:10,000), RRM1 (D12F12) XP Rabbit mAb #8637 (Cell Signaling Technology; 1:5,000), rabbit polyclonal to thymidine phosphorylase (ab69120) (Abcam; 0.4  $\mu$ g/ml), anti-thymidine kinase 1 (EPR3193) antibody (ab76495) (Abcam; 1:50,000), dNT-1 (C-10): sc-390041 (Santa Cruz Biotechnology; 1:100), anti-thymidylate synthase, clone TS106 (MAB4130) (EMD Millipore; 1:5,000) or GAPDH (D16H11) XP Rabbit mAb #5174 (Cell Signaling Technology; 1:5,000) for 2 h at room temperature. The primary antibodies were diluted with Can Get Signal Solution 1 (Toyobo Co., Ltd.). The membranes were then washed with Dako Washing Buffer (Agilent Technologies, Inc.) and incubated with Goat anti-Mouse IgG, Peroxidase Conjugated, heavy chain + light chain (AP124P) (EMD Millipore) or Goat anti-Rabbit IgG, Peroxidase Conjugate (AP132P) (EMD Millipore) diluted to 1:25,000 with Can Get Signal Solution 2 (Toyobo Co., Ltd.) for 1 h at room temperature. Immunoreactive proteins were visualized with the ImmunoStar LD reagent (FUJIFILM Wako Pure Chemical Corporation), and images were captured using a GeneGnome HR system (Syngene Europe). Western blot analysis was repeated at least three times.

**3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay for the effects of 5-FU, raltitrexed or tipiracil.** A total of 5x10<sup>3</sup> cells were seeded into each well of 96-well plates and cultured for 24 h. The cells were treated with 5-FU, raltitrexed or tipiracil for 72 h, and the medium was replaced with 100  $\mu$ l of a 0.5 mg/ml solution of MTT (Sigma-Aldrich; Merck KGaA). The plates were then incubated for 4 h at 37°C. The MTT solution was replaced with 100  $\mu$ l of dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Corporation), and the absorbance at 540 nm was measured using a Sunrise Rainbow RC-R (Tecan Group Ltd. Männedorf, Switzerland). Eight wells were used for each condition.

**Statistical analyses.** The mean half maximal inhibitory concentration (IC<sub>50</sub>) values were calculated based on each result of MTT assays using the Graphpad Prism 9 software program (GraphPad Software, Inc.) presented as the mean, 95% confidence interval (CI). The results of western blot analysis were semi-quantified by dividing by the GAPDH expression and normalized by dividing by an appropriate normalizer and then presented as the mean  $\pm$  standard error (SE). The significance of differences was determined by two-group comparisons using unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Sensitivity to 5-FU and changes in the expression of the enzymes for 5-FU metabolism in breast cancer cells.** MCF-7/5-FUR cells showed an IC<sub>50</sub> of 140.2 (95% CI: 104.3-188.8)  $\mu$ M, which represented a 36.6-fold increased resistance compared with parental MCF-7 cells (IC<sub>50</sub>: 4.79  $\mu$ M, 95% CI: 4.12-5.55) (Fig. 2A). A western blot analysis showed decreased OPRT (1.21 $\pm$ 0.024 to 0.51 $\pm$ 0.12, P<0.05), RR (1.19 $\pm$ 0.026 to 0.39 $\pm$ 0.0022, P<0.01) and TK (0.94 $\pm$ 0.077 to 0.46 $\pm$ 0.063, P=0.051) levels and increased NT (0.40 $\pm$ 0.11 to 2.86 $\pm$ 0.66, P=0.09) and TS (0.21 $\pm$ 0.047 to 4.26 $\pm$ 0.97, P=0.07) levels in

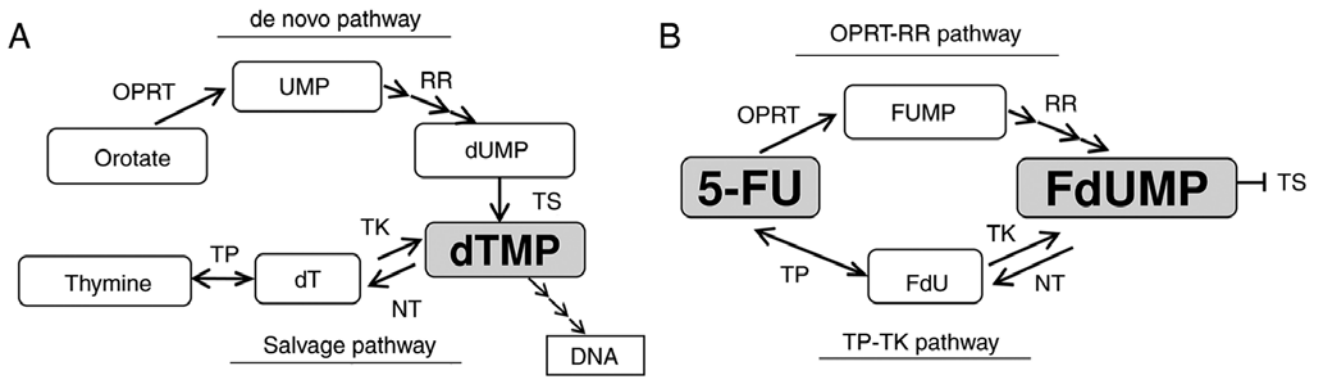


Figure 1. Diagram of the thymidylate (dTMP) synthesis and 5-FU metabolism. (A) The synthesis pathway of dTMP. (B) The metabolism of 5-FU. 5-FU, 5-fluorouracil; dT, thymidine; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FdU, fluoro-deoxyuridine; FdUMP, fluoro-deoxyuridine monophosphate; FUMP, fluoro-uridine monophosphate; NT, nucleotidase; OPRT, orotate phosphoribosyl transferase; RR, ribonucleotide reductase; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; UMP, uridine monophosphate.

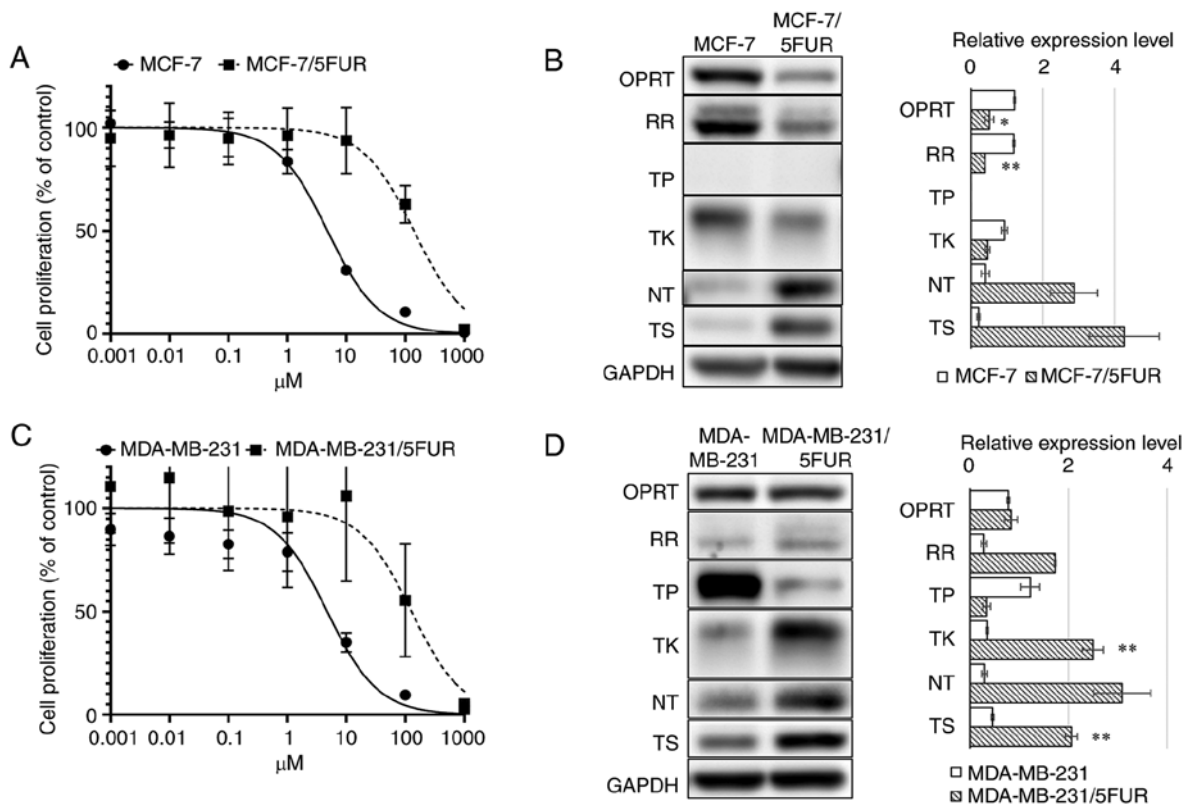


Figure 2. Metabolism for 5-FU and sensitivities to 5-FU. An MTT assay for 5-FU (A) in MCF-7 and MCF-7/5-FUR cells, and (C) in MDA-MB-231 and MDA-MB-231/5-FUR cells. A western blot analysis of the enzymes involved in 5-FU metabolism (B) in MCF-7 and MCF-7/5-FUR cells, and (D) in MDA-MB-231 and MDA-MB-231/5-FUR cells. 5-FU, 5-fluorouracil; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NT, nucleotidase; OPRT, orotate phosphoribosyl transferase; RR, ribonucleotide reductase; TK, thymidine kinase; TP, thymidine phosphorylase; T, thymidylate synthase. \* $P < 0.05$ , \*\* $P < 0.01$  vs. MDA-MB-231.

MCF-7/5-FUR cells compared with parental MCF-7 cells (Fig. 2B).

MDA-MB-231/5-FUR cells showed resistance to 5-FU ( $IC_{50}$ : 127.3  $\mu$ M, 95% CI: 66.9-247.0), which represented a 15.8-fold increased resistance compared with parental MDA-MB-231 cells ( $IC_{50}$ : 4.73  $\mu$ M, 95% CI: 3.49-6.35) (Fig. 2C). A western blot analysis showed decreased TP ( $0.123 \pm 0.19$  to  $0.34 \pm 0.075$ ,  $P = 0.068$ ) levels and increased TK ( $0.35 \pm 0.018$  to  $2.50 \pm 0.21$ ,  $P < 0.01$ ), NT ( $0.30 \pm 0.053$  to  $3.09 \pm 0.58$ ,  $P = 0.05$ ) and TS ( $0.46 \pm 0.026$  to  $2.06 \pm 0.12$ ,  $P < 0.01$ )

levels in MDA-MB-231/5-FUR cells compared with parental MDA-MB-231 cells (Fig. 2D).

These results indicated that these 5-FU-resistant cells showed different changes in the metabolism of 5-FU after the acquisition of resistance to 5-FU.

*Changes in the amount of FdUMP after treatment with 5-FU and the synthesis pathway of FdUMP after the acquisition of resistance to 5-FU.* After treatment with 5-FU, the upper band of TS was detected on a western blot analysis, representing

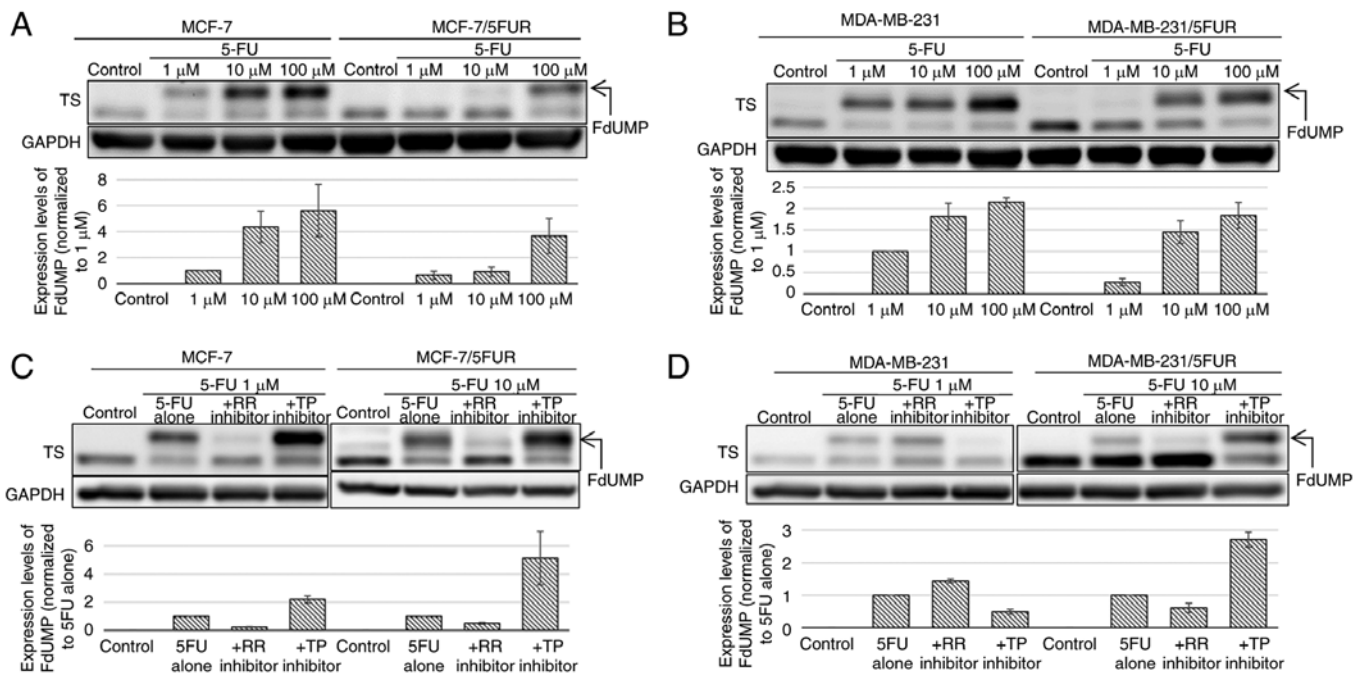


Figure 3. Synthesis of FdUMP after treatment with 5-FU and changes in the amount of FdUMP after treatment with 5-FU when RR or TP was inhibited. A western blot analysis of TS after treatment with 5-FU (A) in MCF-7 and MCF-7/5-FUR cells, and (B) in MDA-MB-231 and MDA-MB-231/5-FUR cells. A western blot analysis of TS after treatment with 5-FU with/without RR inhibitor or TP inhibitor (C) in MCF-7 and MCF-7/5-FUR cells, and (D) in MDA-MB-231 and MDA-MB-231/5-FUR cells. 5-FU, 5-fluorouracil; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RR, ribonucleotide reductase; TP, thymidine phosphorylase; TS, thymidylate synthase.

ternary complexes composed of TS, FdUMP and  $\text{CH}_2\text{THF}$ ; the density of the upper band was correlated with the intracellular concentration of FdUMP (13). In both 5-FU-resistant cell lines, the upper band of TS was decreased compared with the parental cell line: MCF-7/5-FUR (-79.1% at 1  $\mu\text{M}$ ) and MDA-MB-231/5-FUR (-20.2% at 1  $\mu\text{M}$ ) (Fig. 3A and B). These results indicated that the synthesis of FdUMP from 5-FU decreased on acquiring resistance to 5-FU.

Next, we investigated the changes in the amount of FdUMP after treatment with 5-FU combined with an RR or TP inhibitor to clarify through which pathway FdUMP was synthesized. In MCF-7 and MCF-7/5-FUR cells, the upper band of TS was decreased when 5-FU was combined with 1,000  $\mu\text{M}$  of an RR inhibitor (MCF-7: -76.7%, MCF-7/5-FUR: 50.0%), which indicated that FdUMP was synthesized through the OPRT-RR pathway in these cells (Fig. 3C). The upper band of TS was decreased by 51.0% when 5-FU was combined with 1  $\mu\text{M}$  of a TP inhibitor in MDA-MB-231 cells and was decreased by 39.1% when combined with an RR inhibitor in MDA-MB-231/5-FUR cells, which indicated that the synthesis pathway of FdUMP changed from the TP-TK pathway to the OPRT-RR pathway on the acquisition of resistance to 5-FU by these cells (Fig. 3D).

Interestingly, the upper band of TS was increased when 5-FU was combined with a TP inhibitor in MCF-7/5-FUR cells (+412%) as well as in MDA-MB-231/5-FUR cells (+171%), which suggested that synthesized FdUMP was reduced through the TP-TK pathway, and such reduction was inhibited by the TP inhibitor in these cells.

*The survival of MDA-MB-231 and MDA-MB-231/5-FUR cells without TS activity, with no products derived from the de novo*

*pathway detected in these cells.* TS is a target enzyme of FdUMP and essential for the synthesis of dTMP through the *de novo* pathway (14). Therefore, the inhibition of TS leads to cell death if the cell is dependent on *de novo* synthesis of dTMP.

In MCF-7 and MCF-7/5-FUR cells, the  $\text{IC}_{50}$  for the TS inhibitor was 7.00 nM (95% CI: 4.99-8.90) and 8.19 nM (95% CI: 6.50-10.47), respectively (Fig. 4A). The cell growth of MDA-MB-231 and MDA-MB-231/5-FUR cells was not affected despite the presence of more than 1,000 nM of a TS inhibitor (Fig. 4B). These results indicated that MDA-MB-231 and MDA-MB-231/5-FUR cells were not dependent on the *de novo* pathway, with the dTMP in these cells being synthesized mainly through the salvage pathway.

*Reversal of 5-FU resistance in both 5-FU-resistant cell lines by a TP inhibitor.* The TP inhibitor alone did not exert any cytotoxicity in either cell line on an MTT assay (Fig. 5A and B). Interestingly, resistance to 5-FU in 5-FU-resistant cells was completely reversed in MCF-7/5-FUR cells ( $\text{IC}_{50}$ : 219.9  $\mu\text{M}$ , 95% CI: 90.44-555.0 to 0.934  $\mu\text{M}$ , 95% CI: 0.059-0.148) and decreased in MDA-MB-231/5-FUR cells ( $\text{IC}_{50}$ : 157.3  $\mu\text{M}$ , 95% CI: 132.8-186.5 to 30.98  $\mu\text{M}$ , 95% CI: 25.6-37.6) when 1  $\mu\text{M}$  of a TP inhibitor was combined with 5-FU, although the  $\text{IC}_{50}$ s in parental MCF-7 and MDA-MB-231 cells were not so changed (MCF-7:  $\text{IC}_{50}$ : 6.41  $\mu\text{M}$ , 95% CI: 4.50-9.10 to 0.724  $\mu\text{M}$ , 95% CI: 0.337-1.54, MDA-MB-231: 7.95  $\mu\text{M}$ , 95% CI: 0.811-0.990 to 9.60  $\mu\text{M}$ , 95% CI: 8.12-11.4, respectively) (Fig. 5C and D).

These results suggested that the inhibition of TP could reverse resistance to 5-FU in 5-FU-resistant cells, although the TP inhibitor itself did not show any cytotoxic effect.

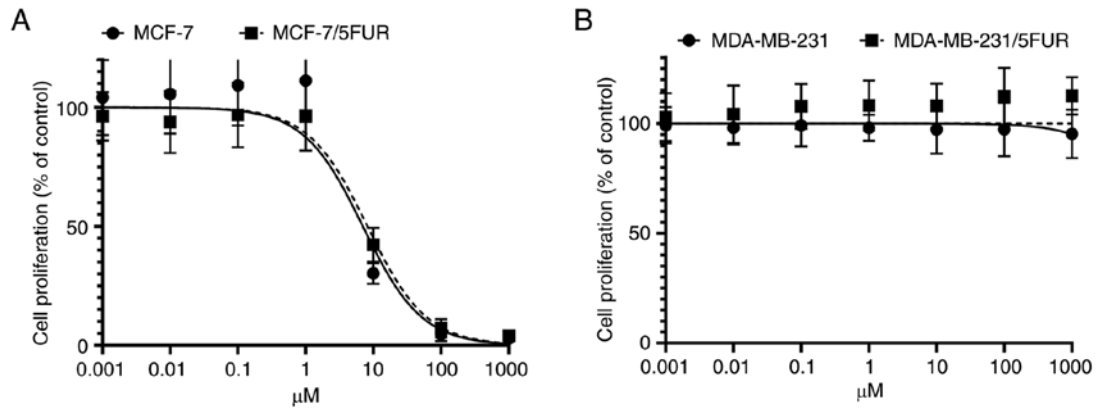


Figure 4. The inhibition of TS by TS inhibitor and the amount of intracellular deoxynucleosides. An MTT assay for TS inhibitor (A) in MCF-7 and MCF-7/5-FUR cells, and (B) in MDA-MB-231 and MDA-MB-231/5-FUR cells. dTDP, deoxythymidine diphosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate.

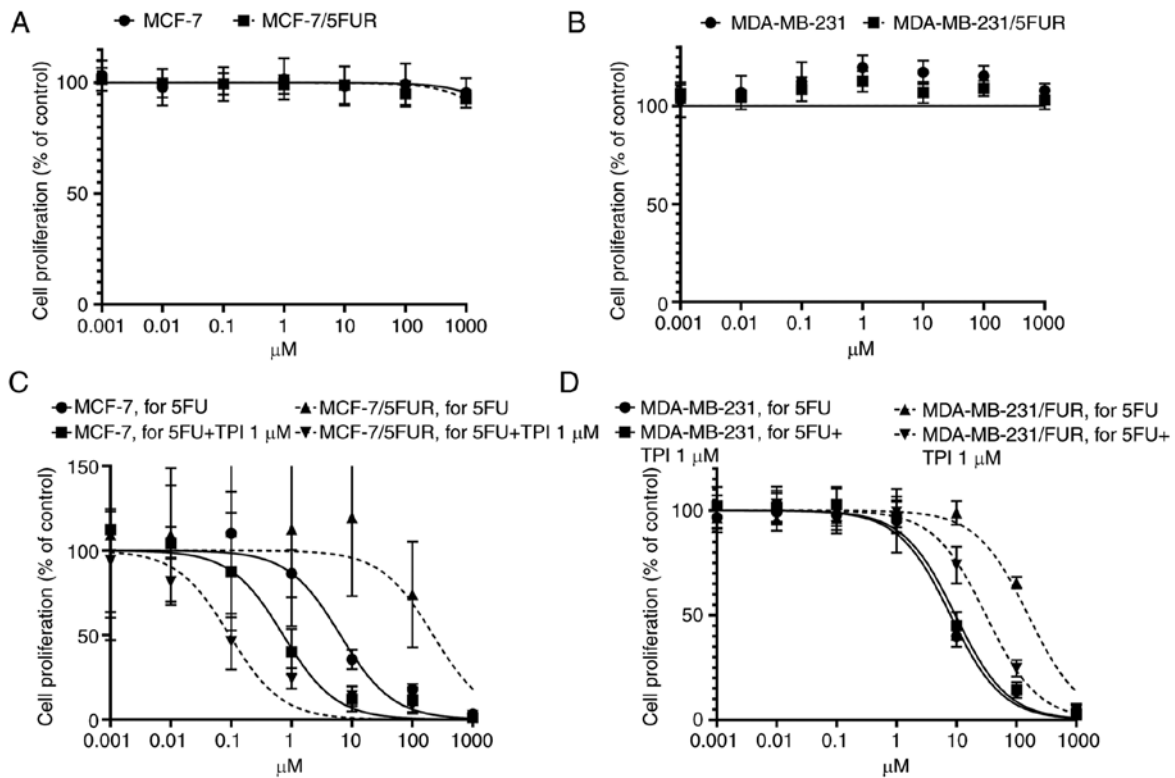


Figure 5. Reversal of resistance to 5-FU by TP inhibitor. An MTT assay (A) for TP inhibitor alone in MCF-7 and MCF-7 cells, (B) for TP inhibitor alone in MDA-MB-231 and MDA-MB-231/5-FUR cells, (C) for 5-FU with/without 1  $\mu$ M of TP inhibitor in MCF-7 and MCF-7/5-FUR cells, and (D) for 5-FU with/without 1  $\mu$ M of TP inhibitor in MDA-MB-231 and MDA-MB-231/5-FUR cells. 5-FU, 5-fluorouracil; TP, thymidine phosphorylase.

## Discussion

In the present study, we clarified that the metabolism of 5-FU differed in each cell line, and the mechanism underlying the resistance to 5-FU also differed in each 5-FU-resistant cancer cell line. In addition, we found that resistance to 5-FU was reversed by the inhibition of TP by a TP inhibitor in both 5-FU-resistant cell lines.

In MCF-7 cells, FdUMP is synthesized only through the OPRT-RR pathway and reduced through the TP-TK pathway. After the acquisition of resistance to 5-FU, the synthesis of FdUMP was decreased by decreased OPRT, leading to

resistance to 5-FU. To maintain a sufficient supply of dTMP, MCF-7/5-FUR cells seemed to increase the reduction of FdUMP, which inhibited the synthesis of dTMP through the *de novo* pathway by decreased TK and increased NT levels. These hypotheses are illustrated in Fig. 6A and B.

However, in MDA-MB-231 cells, FdUMP was synthesized mainly through the TP-TK pathway, although some FdUMP was synthesized through the OPRT-RR pathway. After the acquisition of resistance to 5-FU, the synthesis of FdUMP through the TP-TK pathway disappeared due to the extremely decreased TP levels. The synthesis of dTMP in these cells was mainly dependent on the salvage pathway, and the expression

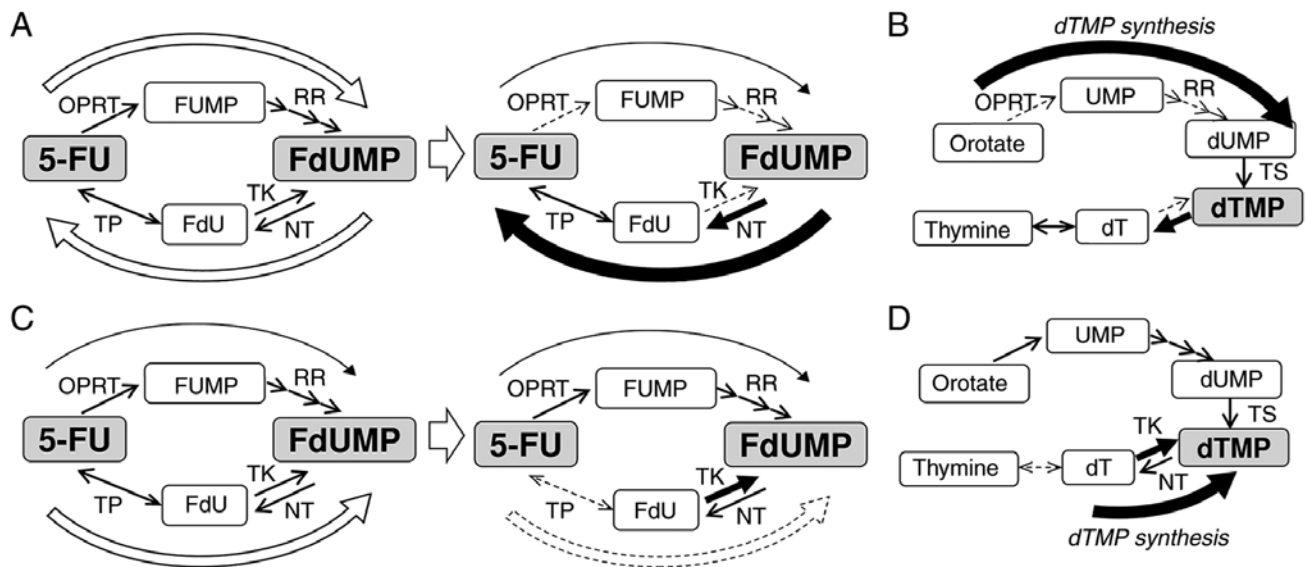


Figure 6. Diagram for the mechanism underlying resistance to 5-FU and the synthesis of dTMP in 5-FU-resistant breast cancer cells. (A) In MCF-7 cells, FdUMP is synthesized through the OPRT-RR pathway and reduced through the TP-TK pathway. After the acquisition of resistance to 5-FU, synthesis of FdUMP was decreased, and reduction of FdUMP was increased. As described in the results, (B) in MCF-7 and MCF-7/5UR cells, dTMP is synthesized only through the *de novo* pathway. (C) In MDA-MB-231 cells, FdUMP was synthesized mainly through the TP-TK pathway, although some FdUMP was synthesized through the OPRT-RR pathway. After the acquisition of resistance to 5-FU, the synthesis of FdUMP through the TP-TK pathway disappeared. (D) In MDA-MB-231 and MDA-MB-231/5-FUR cells, the synthesis of dTMP occurred mainly through the salvage pathway. 5-FU, 5-fluorouracil; dT, thymidine; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FdU, fluoro-deoxyuridine; FdUMP, fluoro-deoxyuridine monophosphate; FUMP, fluoro-uridine monophosphate; NT, nucleotidase; OPRT, orotate phosphoribosyl transferase; RR, ribonucleotide reductase; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; UMP, uridine monophosphate.

of TK seemed to be increased to supply sufficient dTMP through the salvage pathway. These hypotheses are illustrated in Fig. 6C and D.

There have been many reports on the association between resistance to 5-FU and the changes in the expression of the enzymes that lead to the decreased synthesis of FdUMP. For example, decreased OPRT is a predictor of resistance to 5-FU (15-17). However, such changes in the enzymes may also decrease the synthesis of dTMP, which is vital for cell growth. Therefore, the acquisition of resistance to 5-FU is usually accompanied by changes in the enzymes increasing the synthesis of dTMP, which has never been clearly reported previously. In the present study, the reduction of FdUMP through the TP-TK pathway in MCF-7/5-FUR cells seemed to contribute to an increase in the synthesis of dTMP through *de novo* synthesis, and the increased TK levels in MDA-MB-231/5-FUR cells also seemed to contribute to an increase in the synthesis of thymidylate through salvage synthesis. Based on this hypothesis, all enzymes related to the metabolism of 5-FU and dTMP should be examined when we investigate the mechanism of 5-FU.

Tipiracil, the TP inhibitor used in the present study, has already been applied as a component of TAS-102, an anti-cancer drug used practically in metastatic colorectal cancer (18) and gastric cancer (19), and its efficacy and safety in daily practice has already been established. Therefore, combination therapy with 5-FU and a TP inhibitor would be easy to incorporate, and we believe it will be a promising therapy for breast cancer.

We previously reported the mechanism underlying the resistance to 5-FU, focusing on the metabolism of 5-FU and FdUMP using other 5-FU-resistant cell lines. In the present study, in the 5-FU-resistant MKN45/F2R cells established

at our institution, resistance to 5-FU was almost completely reversed by the inhibition of TP like MCF-7/5FUR cells, although sensitivity to 5FU was not changed by the inhibition of TP in parental MKN45 cells, unlike MCF-7 cells (20,21). In contrast, the resistance to 5-FU in 5-FU-resistant SW48 and LS174T colon cancer cells, which were also established in our institution, was not reversed by the inhibition of TP although FdUMP in these cells was synthesized through the TP-TK pathway, like the MDA-MB-231/5FUR cells in the present study (22). To our knowledge, this is the first report describing the enhancement of the efficacy of 5-FU in parental cells and the reversal of resistance to 5-FU in cells in which FdUMP was synthesized through the TP-TK pathway. These results suggest that a predictive marker of the efficacy of TP inhibitor should be established.

Several limitations associated with the present study warrant mention. In this study, an *in vivo* experiment was not conducted, so the reversal of resistance to 5-FU by a TP inhibitor *in vivo* is unclear. In addition, predictive markers of the efficacy of TP inhibitors must be developed in order to apply combination therapy with 5-FU and a TP inhibitor in daily practice, as described above. Furthermore, we did not perform high-throughput sequencing or metabolomic analysis for the cells, and the mutational statuses, gene expression profiles and metabolic profiles in parental cells and 5FU-resistant cells were not compared.

In conclusion, we elucidated the differences in the mechanisms underlying resistance to 5-FU among cell lines. In addition, we observed the reversal of resistance of 5-FU in 5-FU-resistant cells by treatment with a TP inhibitor. Further investigations regarding the mechanism underlying resistance to 5-FU in other 5-FU-resistant cell lines and predictive

markers for the reversal of resistance to 5-FU by TP inhibitors are required. Such combination therapy involving 5-FU and a TP inhibitor will hopefully be able to be applied in clinical practice in the future.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

RM and MF designed the study. RM carried out the study, and wrote the manuscript. RM and MF confirm the authenticity of all the raw data. JU, YT and YN participated in the design and helped draft the manuscript. MF represented our division, and supervised the study and the writing of the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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