Inhibition of dUTPase Induces Synthetic Lethality with Thymidylate Synthase–Targeted Therapies in Non–Small Cell Lung Cancer

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Abstract

Chemotherapies that target thymidylate synthase (TS) continue to see considerable clinical expansion in non–small cell lung cancer (NSCLC). One drawback to TS-targeted therapies is drug resistance and subsequent treatment failure. Novel therapeutic and biomarker-driven strategies are urgently needed. The enzyme deoxyuridine triphosphate nucleotidohydrolase (dUTPase) is reported to protect tumor cells from aberrant misincorporation of uracil during TS inhibition. The goal of this study was to investigate the expression and significance of dUTPase in mediating response to TS-targeted agents in NSCLC. The expression of dUTPase in NSCLC cell lines and clinical specimens was measured by quantitative real-time reverse transcriptase PCR and immunohistochemistry. Using a validated RNA interference approach, dUTPase was effectively silenced in a panel of NSCLC cell lines and response to the fluoropyrimidine fluorodeoxyuridine (FUdR) and the antifolate pemetrexed was analyzed using growth inhibition and clonogenic assays. Apoptosis was analyzed by flow cytometry. Significant variation in the quantity and cellular expression of dUTPase was observed, including clear evidence of overexpression in NSCLC cell line models and tumor specimens at the mRNA and protein level. RNA interference–mediated silencing of dUTPase significantly sensitized NSCLC cells to growth inhibition induced by FUdR and pemetrexed. This sensitization was accompanied by a significant expansion of intracellular dUTP pools and significant decreases in NSCLC cell viability evaluated by clonogenicity and apoptotic analyses. Together, these results strongly suggest that uracil misincorporation is a potent determinant of cytotoxicity to TS inhibition in NSCLC and that inhibition of dUTPase is a mechanism-based therapeutic approach to significantly enhance the efficacy of TS-targeted chemotherapeutic agents.

Introduction

In 2010, more than 222,500 patients were diagnosed with lung cancer and more than 157,000 lung cancer patients succumbed to their disease, making this the deadliest malignancy in the United States (1). Non–small cell lung cancer (NSCLC) accounts for 85% of cases, and most patients are diagnosed at an advanced stage and require chemotherapy to control their disease (2). However, novel therapeutics in NSCLC has resulted in only minor improvements in outcomes for the majority of patients and novel therapeutic strategies are urgently needed.

For over 50 years, chemotherapeutic agents that target thymidylate metabolism have seen widespread clinical implementation in a wide range of neoplasias. The production of dTMP from dUMP is catalyzed by the enzyme thymidylate synthase (TS), encoded by the TYMS gene, and represents the sole source of de novo cellular thymidylate for DNA synthesis (3). Inhibition of TS depletes thymidylate pools, inducing a thymineless state and growth arrest (4–6). Two classes of therapeutic agents that target thymidylate metabolism via TS inhibition include the fluoropyrimidines and antifolates. The fluoropyrimidines 5-fluorouracil (5-FU) and fluorodeoxyuridine (FUdR) were the first TS inhibitors to show clinical efficacy (7, 8). The antifolate class includes agents such as raltitrexed (9) and pemetrexed (10) that also function primarily through TS inhibition (11).
treatment (13–15) and is undergoing significant clinical expansion. Although fluoropyrimidine-based regimens are not widely implemented in NSCLC in the United States, this class is seeing expanding application in Japan and Europe. Tegafur-uracil (UFT) and S-1 are oral 5-FU derivatives containing inhibitors of the 5-FU catabolic enzyme dihydroxypyrimidine dehydrogenase that is often overexpressed in NSCLC.

One drawback to TS-directed therapies is the presence of intrinsic or acquired resistance. Because therapeutic response is difficult to predict using clinical and pathologic factors, considerable effort has been directed toward understanding mechanisms of drug action and identifying biomarkers to predict response to therapy. An increasing number of studies have shown that elevated TS expression is associated with resistance to fluoropyrimidine-based therapy in colorectal cancer (16–20) and S-1 (21) and UPT (22) in NSCLC. Accumulating evidence also indicates that TS overexpression is a determinant of sensitivity to pemetrexed (23–29). However, benefits from TS-targeted chemotherapy in NSCLC seems to have plateaued, and their continued clinical success may depend on our ability to correctly administer these agents following biomarker-driven patient selection (30).

A less appreciated component of cell death induced by TS inhibition is aberrant uracil incorporation into DNA resulting from accumulation of the cytotoxic nucleotide intermediate dUTP. When dUTP pools accumulate, DNA polymerases use dUTP in place of TTP during DNA synthesis, activating iterative rounds of uracil repair and reincorporation due to continued TTP depletion, resulting in extensive DNA damage and cell death. Although the depletion of dTMP and the accumulation of dUMP is common in cancer cell lines undergoing TS inhibition (31, 32), the ability to accumulate dUTP varies dramatically. The enzyme deoxyuridine triphosphate nucleotidohydrolase (dUTPase), encoded by the DUT gene, is the sole regulator of cellular dUTP pools, hydrolyzing dUTP to dUMP, thereby eliminating dUTP from the DNA biosynthetic pathway. We previously reported that overexpression of dUTPase could protect colorectal and breast cancer cells from cytotoxicity induced from dUTP pool expansion and subsequent uracil misincorporation into DNA induced during TS inhibition (33, 34). In addition, dUTPase overexpression in tumor specimens is associated with resistance to 5-FU in colorectal cancer (34, 35). Numerous solid tumors overexpress dUTPase, abrogating the potential for dUTP pool expansion and cytotoxicity induced by the uracil–DNA misincorporation pathway. However, if dUTPase activity could be impaired in the presence of TS inhibition, the combined cytotoxicity from concomitant TTP pool depletion and uracil misincorporation would result in enhanced DNA damage and cell death (Supplementary Fig. S1).

As TS-targeted therapies are seeing considerable clinical expansion in NSCLC, the goal of this study was to evaluate the expression of dUTPase in NSCLC tumor cell lines and clinical specimens and to use in vitro models to evaluate the role of dUTPase in determining sensitivity to 2 class-specific TS-targeted therapies.

Materials and Methods

Additional details can be found in the Supplementary Methods.

Compounds and reagents

FUDR and propidium iodide were purchased from Sigma. Pemetrexed was purchased from LC Laboratories. CellTiter96 Aqueous MTS was purchased from Promega. Halt protease/phosphatase inhibitor was purchased from Thermo Scientific.

Cell culture

The NSCLC cell lines H1299 (metastatic lung adenocarcinoma), H358 (bronchioalveolar carcinoma), and H460 (large cell carcinoma), A549, H1563, H2228, H522, and H23 (adenocarcinoma) were purchased directly from American Type Culture Collection and were authenticated before receipt. Cells were maintained in folate-depleted RPMI supplemented with 25 nmol/L 5-formyltetrahydrofolate (Invitrogen), 10% FBS (Gemini) with penicillin/streptomycin, and l-glutamine (Invitrogen) in a humidified incubator (Forma) at 37°C with 5% CO₂.

Growth inhibition assay

Cells were seeded in 96-well plates at a density of 1,500 cells per well with continuous exposure to increasing concentrations of drug for 72 hours and quantified by the CellTiter96 Aqueous MTS (Promega) assay as previously described (36).

Colony formation assay

The colony formation assay was carried out as previously described (37). Briefly, cells were seeded at a density of approximately 75 cells per well in a 24-well plate and following a 24-hour drug exposure, media was removed and replaced with drug-free medium and cells allowed to grow for 7 to 14 days. Colonies were fixed, stained with crystal violet, scanned, and colonies with more than 100 cells counted. Drug-treated samples were compared directly with vehicle-treated controls.

Flow cytometric/Sub-G₁ analysis

Apoptosis was determined by flow cytometric analysis of DNA content using propidium iodide as previously described (38). Cells were harvested at the indicated time, fixed, stained, and analyzed using an EPICS ELITE flow cytometer with cell populations quantified using Expo32 software (Beckman Coulter). Cells with DNA content less than 1 were considered apoptotic.

Immunoblotting

Immunoblotting was done as previously described (38). Cell lysates were prepared using radioimmunoprecipitation assay buffer with Protease and Phosphatase Inhibitors.
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RNA interference
RNA interference was done as previously described using the siDUT/230 (si-DUT) small interfering RNA (siRNA) oligos (sense; 5′-CGG ACA UUC AGA UAG CGC UdTT; and antisense; 5′-AGC GCU UAU CUG AAU GUC CGd TT (33, 37). Cells were treated with 25 nM si-DUT complexed with Lipofectamine RNAiMAX (Invitrogen). A nontargeted scrambled control siRNA (si-SCR) was used at equimolar concentration. Transfection complexes were removed after 12 hours and dUTPase silencing was confirmed by quantitative (q) real-time reverse transcriptase PCR (RT-PCR) and Western blotting. No significant toxicity or reduction in plating efficiency was observed with either the si-SCR or si-DUT transfections in any of the cell lines.

Quantitative real-time RT-PCR
RNA was isolated using TRizol according to manufacturer’s instructions (Invitrogen). For clinical specimens, tumor specimens were fresh frozen at the time of surgery and stored at −80°C until RNA extraction. cDNA was reverse transcribed and analyzed using PerfeCta SYBR Green (Quanta Biosciences Inc.) on an Applied Biosystems 7500 PCR System (Applied Biosystems). For in vitro analyses, DUT and TYMS were normalized to GAPDH or ACTB (β-actin) and quantified using comparative C_t methodology (39). For clinical specimens, parallel determinations were done for TYMS and DUT. ACTB was used for normalization and the denominator for the ratio used to determine relative gene expression (40).

Nucleotide pool determinations
Cells were treated with FUdR or pemetrexed for 24 hours, harvested, and 2 × 10^5 cells were analyzed for nucleotide pool content using a 96-well fluorescence-based assay developed in our laboratory and as described previously (41). The assay measures the Taq polymerase-mediated incorporation of a limiting dNTP (TTP) into newly synthesized DNA and was modified to distinguish between TTP and dUTP by parallel reactions with and without a pre-incubation step including 10 ng of recombinant dUTPase (41). Fluorescence generated in the presence of dUTPase represented the TTP pool, whereas undigested extracts represented the combined dUTP and TTP pools. dUTP was quantified by subtracting the results of extracts treated with dUTPase from untreated extracts and presented as pmol TTP/10^6 cells as determined by standard curve.

Patients and specimens
Deidentified clinical specimens were obtained from 35 histopathologically confirmed stage II NSCLC patients at the University of California Davis Cancer Center. All 35 patients had formalin-fixed, paraffin-embedded (FFPE) tissue available for immunohistochemistry (IHC) and 32 had corresponding RNA extracted from microdissected primary tumor biopsy material. Of the 32 patients with both FFPE and RNA available, 21% had squamous cell carcinoma (SCC), 50% had adenocarcinoma, 6% had large cell carcinoma, and 6% had bronchi alveolar carcinoma. The remaining 17% of patients had neuroendocrine large-cell type, carcinoid, and large cell carcinoma undifferentiated. In these 32 patients, the median age was 68 and 81% had a history of smoking. Data and tissue collection were in accordance with the regulations of the local ethics committee and Institutional Review Board. In addition, 6 normal lung tissue FFPE specimens were available.

Immunohistochemistry
IHC was conducted at the University of Southern California Immunohistochemistry Clinical Laboratory using the DUT415 (42) dUTPase monoclonal antibody (2 μg/mL; ref. 43) and the TS polyclonal antibody (1 μg/mL) using methods previously described (34). Slides were reviewed by 2 independent observers at the University of Southern California Norris Comprehensive Cancer Center (RDL and PMW). The scoring field was ×100, and the whole of each tissue was scored. Staining intensity was graded as 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). Staining extent was graded by mean percentage of cells staining positive. The mean tumor expression index was calculated by multiplying the percent positive and intensity scores for each slide (semiquantitative system; range: 0–300).

Statistical analyses
Quantitative data are presented as mean ± SEM and analyzed by Student 2-tailed t test in which a P value of 0.05 or less was considered statistically significant and *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 (GraphPad).

Results
dUTPase and TS show wide-ranging expression in NSCLC cell lines
The expression of dUTPase protein and mRNA was analyzed in 8 NSCLC cell lines during log-phase growth. The NSCLC cells show an 38-fold range in nuclear dUTPase expression and a 18-fold range in mitochondrial dUTPase protein expression. Subsequent measurement of DUT mRNA by qRT-PCR revealed a similar pattern of mRNA expression to the protein expression but with a reduced range of 6.5-fold. TS expression showed a similar range in protein expression as observed with dUTPase of approximately 16-fold. TS protein also showed a similar range in protein expression as observed with dUTPase expression and a 18-fold range in mitochondrial TS protein. TYMS protein expression was relatively constant, showing a similar range in protein expression as observed with dUTPase expression and a 18-fold range in mitochondrial TS protein. TYMS mRNA although the range of mRNA expression observed was less than the protein at 10-fold. TYMS and DUT mRNA expression exhibited a correlation in these 8 NSCLC cell lines (R^2 = 0.6; P = 0.027, Fig. 1).
Aberrant dUTPase expression in NSCLC clinical specimens

Primary tumor specimens were obtained from 35 histopathologically confirmed NSCLC patients (stage II) and included the primary histologic subtypes of adenocarcinoma and SCC. To investigate the expression and intracellular localization of dUTPase in NSCLC, IHC on FFPE tumor tissue and qRT-PCR on tumor-extracted RNA was done. Thirty-five specimens were available for IHC with 32 of these having matched RNA available for qRT-PCR. Expression of dUTPase protein was detected in 89% of the tumor specimens and 0% of the normal lung specimens. dUTPase expression varied significantly in the quantity of expression and intracellular localization. Of the specimens that stained positive for dUTPase, 51.4% exhibited both nuclear and cytoplasmic expression, 8.6% were nuclear-only and 28.6% were cytoplasmic staining only. Figure 2A shows a typical dUTPase staining pattern for replicating cells in human palatine tonsil. In contrast, Fig. 2B shows a lack of dUTPase staining in a representative normal lung specimen. In Fig. 2C, dUTPase is highly abundant in both the NSCLC cell cytoplasm and nucleus, whereas the surrounding stromal cells exhibit no dUTPase expression. In contrast, in Fig. 2D predominantly cytoplasmic dUTPase expression is observed. Fig. 2E and F illustrate NSCLC specimens with weak staining and intense staining for dUTPase with IHC scores of 5 and 270, respectively. Overall, there was clear evidence of elevated dUTPase expression at both the nuclear and mitochondrial level in the tumor tissues compared with the normal and surrounding nontumor tissue. The median dUTPase IHC score was 100 (range: 0–300). Seventy-seven percent of specimens (27 of 35) exhibited a dUTPase IHC score higher than 10. Although the number of clinical specimens is limited, the analysis of dUTPase expression in NSCLC histologic subtypes revealed a significant difference in expression between...
SCC versus adenocarcinoma. dUTPase IHC score above the median was found in 57% of SCC as compared with 25% of adenocarcinoma (P = 0.01), although the overall range of expression in adenocarcinoma and SCC were similar (0–300 and 0–280, respectively).

The mRNA encoding dUTPase is aberrantly expressed in clinical specimens of NSCLC

Following microdissection, RNA was extracted and processed from the 32 tumor specimens, and DUT gene expression relative to ACTB (β-actin) was determined by qRT-PCR alongside TYMS (Fig. 2G). A range in DUT mRNA expression of 7-fold was observed across all 32 NSCLC specimens, similar to the range observed in the cell line panel. Subgroup analysis of the histologic subtypes revealed a narrow 2-fold range in DUT expression in SCC compared with the wider 5-fold variation observed in adenocarcinoma (Fig. 2H). Importantly, DUT mRNA correlated with the protein expression detected by IHC (R² = 0.51; P = 0.027).

Expression of dUTPase does not correlate with TS in NSCLC

The pattern of TS expression in the NSCLC specimens was also analyzed by IHC and qRT-PCR. The median IHC score for TS was 100 (range 0–300) with 94% of tumor specimens staining positive (Supplementary Fig. S2). TYMS mRNA expression correlated with TS IHC (R² = 0.40; P = 0.035). When all histologic subtypes were combined, there was no correlation between DUT and TYMS mRNA expression (R² = 0.01; P = 0.56; n = 32; Fig. 2G) or protein expression measured by IHC (R² = 0.1; P = 0.1; n = 35), suggesting that these genes are not coregulated in NSCLC. The median IHC score for TS was higher in SCC than adenocarcinoma with scores of 200 and 100, respectively, with TS IHC score above the median found...
in 71% of SCC as compared with 31% of adenocarcinoma. Analysis of TYMS mRNA also showed a higher median expression in SCC when compared with adenocarcinoma consistent with previous observations (27). However, as \( \frac{\text{DUT}}{\text{si-SCR}} \) expressed as a percentage compared with just 2.8-fold in SCC (\( P = 0.006; \) Fig. 2I).

**TS, but not dUTPase, protein expression is induced in response to fluoropyrimidine and antifolate treatment**

The acute induction of TS protein following treatment with TS-inhibiting agents has been reported in a variety of neoplasias and proposed as a mechanism of resistance to treatment. However, the expression of dUTPase in response to TS inhibition in NSCLC cells has not been evaluated. Four NSCLC cell lines were exposed to increasing concentrations of FUdR and pemetrexed and protein expression of TS and dUTPase was measured. FUdR treatment was characterized by the appearance of the increased molecular weight ternary TS complex. However, with the exception of the A549 cells (low TS expressing), all cell lines showed detectable expression of free (uninhibited) TS enzyme as well as the TS-inhibited complex. These cells also showed induction of TS protein ranging from 2- to 5-fold higher than vehicle controls after FUdR treatment. Similarly, treatment with pemetrexed resulted in an increase in TS protein expression between 2- and 10-fold higher than vehicle controls and was observed in all 4 cell lines. No evidence of increased dUTPase expression was observed in response to FUdR or pemetrexed treatment (Fig. 3, left).

![Figure 3](image-url)

**Figure 3.** Evaluation of dUTPase expression in response to TS inhibitor treatment and validation of siRNA-mediated silencing. Left column: dUTPase and TS protein expression was evaluated in response to FUdR and pemetrexed treatment. Western blot bands were quantified by ImageJ64 and normalized to their respective \( \beta \)-tubulin and compared with vehicle-treated controls. Middle column, 4 NSCLC cell lines were transfected with either dUTPase-targeted siRNA (si-DUT), scrambled control siRNA (si-SCR), or TS-targeted siRNA at 25 mmol/L. dUTPase and TS expression was measured by Western blotting and bands were quantified by ImageJ64 and normalized to \( \beta \)-tubulin and compared with si-SCR-transfected control. Numbers below bands correspond to the fold change compared with the si-SCR-transfected control. Right column, \( DUT \) and TYMS mRNA expression was analyzed by qRT-PCR, normalized to GAPDH, and expressed as a percentage compared with si-SCR-transfected control. **,** \( P \leq 0.01; \) ***,** \( P \leq 0.001. \)
Efficient dUTPase gene silencing in NSCLC cells

Having confirmed aberrant dUTPase overexpression in NSCLC, the influence of dUTPase expression on response to TS-targeted chemotherapy was analyzed. Using a previously validated siRNA targeting dUTPase (si-DUT; ref. 37), 4 NSCLC cell lines were treated with 25 nmol/L of si-DUT. The specificity of the siRNA was confirmed by parallel transfections with both 25 nmol/L of a nontargeted scrambled control siRNA (si-SCR) and an additional TS-directed control siRNA (si-TS). In all 4 NSCLC cell lines, efficient and specific dUTPase silencing with si-DUT was observed with more than 80% reduction in expression at the protein and mRNA levels measured by qRT-PCR and Western blotting at 24 and 48 hours, respectively (Fig. 3, middle and right). Importantly, siRNA-mediated dUTPase silencing had no effect on TS mRNA or protein expression and the transfection methodology had no detectable effect on plating efficiency or subsequent cell viability.

Inhibition of dUTPase in NSCLC cells induces synthetic lethality in combination with fluoropyrimidine and antifolate-based TS inhibitors

The growth inhibitory and cytotoxic effects of FuDR and pemetrexed in NSCLC cells were analyzed in the presence and absence of dUTPase silencing. Three of the 4 NSCLC cell lines were inherently resistant to the growth-inhibitory effects of FuDR, with the H460 cell line being the exception. When dUTPase expression was suppressed in si-DUT–transfected cells, a significant increase in growth inhibition was observed in the A549, H1299, and H358 cells in response to increasing FuDR concentrations compared with the si-SCR–transfected cells. In H460 cells, sensitivity to FuDR was only enhanced at elevated concentrations in which the si-SCR–transfected cells seemed to recover, possibly due to salvage of nucleotides from apoptotic cells (Fig. 4). Interestingly, NSCLC cells were more sensitive to the growth inhibitory effects of pemetrexed, with dose-dependent increases in growth inhibition observed. However, although the concentration at which growth inhibition becomes apparent was similar for si-DUT- and si-SCR–transfected cells, silencing of dUTPase showed a significant overall increase in growth inhibition at all subsequent concentrations analyzed (Fig. 4). Calcein–AM cell viability analysis was also done and confirmed the reduction in cell proliferation associated with dUTPase silencing following treatment with pemetrexed. The inclusion of 10 μmol/L thymidine in culture media rescued the growth inhibitory effects of pemetrexed in both si-SCR- and si-DUT–transfected cells to that of vehicle-treated controls (Supplementary Fig. S3), indicating that the growth inhibitory effects of pemetrexed in these NSCLC cells is mediated primarily through TS inhibition and that the sensitization observed with dUTPase silencing is dependent on TS inhibition.

dUTPase silencing suppresses the ability of NSCLC cells to survive and recover from transient exposure to FuDR or pemetrexed

Colony-forming assays were carried out to assess the ability of 4 NSCLC cell lines to recover following a transient 24-hour exposure to increasing concentrations of FuDR or pemetrexed. A dose-dependent decrease in the colony formation capacity was observed in NSCLC cells treated with increasing concentrations of FuDR. However, suppression of dUTPase induced highly significant reductions in colonies formed at all concentrations of FuDR. Similarly, treatment with increasing concentrations of pemetrexed reduced colony-forming capacity to varying degrees in NSCLC cells, ranging from 30% up to 75% at the highest concentration of 2.5 μmol/L analyzed. However, in all cell lines, suppression of dUTPase conferred a highly significant reduction in colony-forming capacity in NSCLC cells following pemetrexed treatment at virtually all concentrations (Fig. 5). These results indicated that inhibition of dUTPase greatly diminishes the ability of NSCLC cells to survive and recover from transient exposure to a TS-targeted therapy.

dUTPase silencing enhances the expansion of dUTP pools during TS inhibition

A novel fluorescence-based assay was used to measure the concentration of dUTP in NSCLC cells treated with 0.5 μmol/L FuDR and 0.25 μmol/L pemetrexed. In si-SCR–transfected A549 cells, treatment with FuDR and pemetrexed significantly reduced TTP pools from 19 pmol/10^6 in vehicle-treated cells to 6.5 pmol and less than 1 pmol/10^6 cells, respectively. FuDR treatment also induced expansion of the dUTP pool to 23.5 pmol/10^6 cells with pemetrexed increasing dUTP pools to 4 pmol/10^6 cells. However, in si-DUT–transfected cells treated with FuDR, TTP was reduced to less than 0.1 pmol/10^6 cells and dUTP pools were increased to 39 pmol/10^6 cells. Following pemetrexed treatment, TTP pools were also reduced to less than 0.1 pmol/10^6 cells and dUTP pools increased to 21 pmol/10^6 cells (Fig. 6A). In H460 si-SCR–transfected cells, treatment with FuDR or pemetrexed depleted TTP from 24 pmol/10^6 in vehicle-treated cells to less than 1 pmol/10^6 cells with less than 0.1 pmol/10^6 cells of dUTP detected. However, in vehicle-treated si-DUT–transfected cells, approximately 20 and 5 pmol/10^6 cells of TTP and dUTP, respectively, could be detected. In si-DUT–transfected cells treated with FuDR and pemetrexed, the dUTP pool expanded significantly to more than 15 pmol/10^6 cells, whereas the TTP pool was significantly reduced from 24 to less than 5 pmol/10^6 cells (Fig. 6A). These data confirmed that silencing dUTPase activity results in significant increases in dUTP pool expansion during TS inhibition in NSCLC.

dUTPase silencing induces apoptosis in NSCLC cells following exposure to FuDR and pemetrexed

Flow cytometry was used to assess whether silencing dUTPase was effective at inducing apoptosis with FuDR
Figure 4. dUTPase silencing enhances NSCLC sensitivity to FUdR and pemetrexed. Growth inhibition was determined by MTS assay for 72 hours. Four NSCLC cell lines were transfected with either si-SCR or si-DUT for 24 hours and subsequently exposed to increasing doses of FUdR (left column) or pemetrexed (right column). Data points represent mean ± SEM percent growth inhibition (n = 3 experiments) compared with vehicle-treated controls at 100%. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
si-DUT silencing reduces the colony-forming capacity of NSCLC cells following transient exposure to FUdR and pemetrexed. Four NSCLC cell lines were treated with increasing concentrations of FUdR (left column) or pemetrexed (right column) for 24 hours and media subsequently replaced with drug-free media for 12 to 15 days. Data are presented as mean ± SEM percentage colony formation compared with vehicle-treated control (n = 3). Representative images at selected concentrations illustrate key differences in colony-forming capacity between si-DUT and si-SCR-transfected cells following treatment. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

or pemetrexed treatment. Two NSCLC cell lines were transfected with si-DUT or si-SCR and then treated with increasing concentrations of FUdR or pemetrexed. Both the A549 and H358 cell lines transfected with si-DUT showed significant increases in the induction of apoptosis following treatment with FUdR or pemetrexed across the concentration ranges tested. Specifically, increases in apoptosis of approximately 3- to 5-fold were observed in
si-DUT–transfected cells treated with FUdR compared with si-SCR–transfected cells. Similarly, following treatment with pemetrexed, increases of approximately 2- to 4-fold in apoptosis were noted with si-DUT compared with si-SCR–transfected cells (Fig. 6B). Similar data were obtained for additional cell lines analyzed (Supplementary Fig. S4).

Discussion

Inhibitors of thymidylate metabolism represent an important class of antineoplastic agents used for the treatment of head and neck, breast, gastrointestinal, and lung cancers. Elevated intratumoral expression of TS is reported to limit the severity of TTP pool depletion leading to resistance to TS inhibitors (29, 44). However, although TTP depletion can be lethal, a prolonged period of continued TS inhibition is typically required for this to occur, and cancer cells can retain viability in a growth-arrested state following 120 hours of continuous exposure to TS inhibitors (4, 31). What is unclear is whether current therapeutic regimens can achieve the sufficient and persistent inhibition of TS necessary to induce tumor cell death, particularly in tumors that overexpress TS. A second, less-understood mechanism of TS inhibitor activity occurs due to the induction of dramatically increased ratios of dUTP to TTP, resulting in the misincorporation of aberrant uracil metabolites into DNA. However, elevated expression of dUTPase will prevent dUTP pool expansion and subsequent DNA damage from uracil–DNA misincorporation and repair, thus contributing to tumor resistance to TS-targeted therapy (32, 45, 46). The results of this study clearly show the presence of dUTPase overexpression that can protect NSCLC cells from uracil–DNA
misincorporation. In addition, this study shows that NSCLC cell lines have an inherent and robust ability to survive transient exposure to both fluoropyrimidine and antifolate-based TS inhibitors. Although transient exposure to a TS inhibitor did induce growth arrest, the level of apoptosis induced in response to treatment was minimal and the majority of NSCLC cells retained viability and resumed proliferation upon drug removal. However, when dUTPase expression was suppressed, cells exhibited an expansion of the dUTP pool and highly significant increases in growth arrest, potent suppression of colony formation, and the induction of cell death. Previous studies in colorectal cancer cells reported a significant increase in DNA damage and a more rapid loss of viability associated with dUTP pool expansion during TS inhibition, an observation that was reversed by forced expression of dUTPase (47, 48). The data presented here would strongly suggest that NSCLC cells are resistant to cytotoxicity induced by transient TS inhibition; however, this resistance can be effectively overcome by a synthetic lethal approach targeting both TS and dUTPase simultaneously and resulting in activation of the uracil–DNA misincorporation pathway.

Tailored treatments strategies based on histopathology and biomarker expression for NSCLC are receiving increased acceptance based on accumulating clinical evidence (27, 49). As pemetrexed-based therapies are seeing rapid clinical expansion, several studies are reporting that patients with non-SCC derive greater clinical benefit from pemetrexed-based therapy (12). Conversely, patients with SCC seem to benefit more from gemcitabine-based therapy, correlating with the increased TS expression recently reported in SCC as a mechanism of resistance to pemetrexed (27, 50). We identified an aberrant pattern of dUTPase overexpression that varies dramatically in NSCLC tumor specimens, both in magnitude and intracellular localization. Despite the limited number of available specimens in this study, our data support previous reports in which TS expression is elevated in SCC compared with adenocarcinoma. In addition, our data also suggest that SCC may have elevated dUTPase expression that may contribute to the lack of clinical benefit observed with pemetrexed-based therapy in SCC. Although there was no significant correlation between the expression of dUTPase and TS in the clinical specimens, there were notable examples with concomitant overexpression of TS and dUTPase at both the mRNA and protein levels. Such patients are likely to be nonresponders to pemetrexed-based therapy. The wide variation in dUTPase expression, observed particularly in adenocarcinoma, suggests that a subset of NSCLC patients treated with a TS-directed therapy will not benefit from the cytotoxic effects of uracil–DNA misincorporation as a result of elevated dUTPase. These preliminary observations warrant evaluation in larger NSCLC patient populations, including those who received pemetrexed-based therapy to evaluate the relationship between dUTPase expression, treatment outcome, and patient prognosis.

Our increasing knowledge of tumor biology, especially in aggressive and treatment-refractory tumors, such as NSCLC, is providing clear evidence that multiple cellular targets need to be neutralized to induce tumor cell death and elicit lasting clinical efficacy. Although TS may be an important determinant of sensitivity to TS-directed therapy, this study strongly suggests that dUTPase overexpression in NSCLC induces resistance to the parallel mechanism of cytotoxicity induced by uracil misincorporation into DNA (37, 46). Targeting dUTPase and thus exploiting this pathway during TS inhibition represents a mechanism-based synthetic lethal combination approach, with the potential to significantly improve the clinical efficacy of this class of chemotherapies. However, it is also recognized that chemotherapeutic agents that inhibit multiple targets within a specific DNA biosynthetic pathway or DNA processing have the potential to increase treatment-related toxicities, and this remains a consideration with the concomitant targeting of TS and dUTPase. However, despite the continued development of technologies to evaluate potential toxicities with novel therapeutics in silico, it remains difficult to accurately predict how such toxicities will manifest themselves until such times as an agent is evaluated in appropriate combination analyses and schedules in vivo. One promising observation to date is that therapeutic combinations that target multiple aspects of DNA synthesis and processing such as those using combinations of platinum agents with fluoropyrimidines or antifolates (5-FU + oxaliplatin or pemetrexed + cisplatin) and fluoropyrimidines with topoisomerase 1 inhibitors (5-FU + irinotecan) and, indeed, combinations of 3 cytotoxic DNA-damaging agents such as FOLFIRINOX (5-FU, irinotecan, and oxaliplatin) can be successfully implemented in patients with good performance status with manageable toxicities and acceptable patient quality of life, while showing additive and synergistic efficacy (12, 51–53). These combination strategies are not only manageable in the majority of patients, they also continue to serve as the most effective foundational regimens in some of the most difficult-to-treat solid tumors, including colorectal, lung, and pancreatic cancers. The continued development of technologies that assist in targeting drug delivery to tumor cells such as those using prodrug, nanomaterials, or immunoconjugates also have the potential to reduce exposure to normal tissues and represent one strategy to maximize antitumor activity while minimizing the increased risk of adverse events (54). The data presented herein strongly support continued efforts to develop inhibitors of dUTPase as a novel therapeutic strategy in NSCLC and other solid tumors that routinely use TS-targeted therapies. The potential broad-reaching clinical benefit of improving these foundational classes of anticancer therapies is worth the inherent challenges.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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