

Small Interfering RNA-Mediated Suppression of dUTPase Sensitizes Cancer Cell Lines to Thymidylate Synthase Inhibition

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ABSTRACT

Uracil misincorporation into DNA and its associated misrepair have been implicated as contributing components of cytotoxicity resulting from treatment with thymidylate synthase inhibitors. dUTPase, which eliminates dUTP from the DNA biosynthetic pathway, opposes uracil misincorporation; therefore, elevation of this enzyme in cancer cells may contribute to drug resistance. To validate the potential of dUTPase as a target for drug development, we used small interfering RNA directed against this enzyme and determined the effects of decreasing levels of dUTPase on sensitivity to the thymidylate synthase (TS) inhibitor fluorodeoxyuridine (FUDR) in human cancer cell lines. Suppression of dUTPase in SW620 and MCF-7 cells resulted in a significant enhancement in dUTP pool expansion after TS inhibition. This shift in nucleotide pool levels was

accompanied by a significant decrease in the FUDR IC_{50}^{72h} (~75-fold for SW620 cells and ~6-fold for MCF-7 cells), a decline in clonogenic survival, and enhanced DNA double strand break formation. In contrast, depletion of dUTPase in HT29 cells did not substantially affect chemosensitivity or the amount of DNA damage incurred despite a 3-fold increase in dUTP pool expansion. This observation implies that the cytotoxic impact of uracil misincorporation may reach a saturation point in HT29 cells and that a further increase in dUTP levels has no additive effect. Together, these results suggest that uracil misincorporation is a potent determinant of cytotoxicity to TS inhibition and indicate that partial inhibition of dUTPase is a viable therapeutic approach to enhance the efficacy of broadly used chemotherapeutic agents that inhibit TS.

Inhibitors of thymidylate biosynthesis, such as the fluoropyrimidines (5-fluorouracil and 5-fluorodeoxyuridine) and the antifolates (methotrexate), have long been used in the treatment of a broad range of neoplastic diseases (Heidelberg et al., 1957). Efforts toward the development of new agents that target this pathway continue to show considerable promise, most recently with the advent of the oral fluoropyrimidine capecitabine (Xeloda) and the multitargeted antifolate pemetrexed (Alimta) (Johnston and Kaye, 2001; Hochster, 2002; Adjei, 2003). Despite their broad use, the clinical efficacy of these agents is hindered by the occurrence of drug resistance, either intrinsic or acquired. Variability of tumor response to these agents is well documented and often correlates with the ability to successfully inhibit the enzyme thymidylate synthase (TS) (Pullarkat et al., 2001; Edler et al., 2002; Longley et al., 2003; Park et al., 2003; Stoecklmaier and Lenz, 2003). Although incomplete TS inhibition is a major component of drug resistance, it has been demonstrated that response is also modulated by downstream biochemical events (Aherne and Browne, 1999; Cohen, 1971; Goulian et al., 1986; Longley et al., 2003).

Cytotoxicity resulting from TS inhibition has largely been attributed to the consequences of cellular TTP depletion, a process termed “thymineless death”. Although the mechanistic basis of cell death initiated by a thymineless state is multifactorial (Cohen, 1971; Barclay et al., 1982; Goulian et al., 1986; Houghton et al., 1998), accumulating evidence suggests that aberrant uracil misincorporation into DNA is a contributing component of toxicity (Aherne and Browne, 1999; Ladner, 2001). Inhibition of TS results in TTP pool depletion and a subsequent accumulation of the precursor dUMP, which may then be phosphorylated to form dUTP. A significant increase of the dUTP/TTP ratio induces misincorporation of uracil into DNA followed by repair initiated by uracil-DNA-glycosylase. If these conditions are sustained, the cell will experience futile cycles of uracil misincorporation followed by base excision repair that will ultimately result in lethal DNA fragmentation (Hochhauser and Weiss, 1978; Goulian et al., 1980a; Sedwick et al., 1981; Ingraham et al., 1986; el-Hajj et al., 1988; Curtin et al., 1991; Gadsden et al., 1993; Ladner, 2001; Tinkelenberg et al., 2002).

The enzyme dUTPase plays a central role in modulating this iterative process. Under normal cellular conditions,

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ABBREVIATIONS: TS, thymidylate synthase; FUDR, fluorodeoxyuridine; siRNA, small interfering RNA; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; PBS, phosphate-buffered saline; PFGE, pulsed-field gel electrophoresis; 5-FU, 5-fluorouracil.

dUTPase catalyzes the hydrolysis of dUTP to dUMP, eliminating dUTP from the DNA biosynthetic pathway. After treatment with TS-directed chemotherapy, dUTPase activity counters dUTP pool accumulation and therefore may prevent misincorporation of uracil into DNA, leading to drug resistance (McIntosh et al., 1992; Canman et al., 1994; Parsels et al., 1995; Ladner et al., 2000). Studies aimed at clarifying the role of dUTPase as a determinant of drug-induced cytotoxicity have shown that resistance to fluorodeoxyuridine (FUdR) in human colon cancer cell line SW620 correlates with elevated levels of dUTPase compared with a control cell line, HT29 (Canman et al., 1993). Further studies indicate that cell lines with ectopically overexpressed *Escherichia coli* dUTPase are more resistant to FUdR-induced strand breaks and exhibit an increased viability over control cells at 24 h after drug exposure (Canman et al., 1994). In agreement with these findings, elevated expression of dUTPase in the nucleus of tumor cells correlates with resistance to 5-FU-based chemotherapy in patients with metastatic colon cancer (Ladner et al., 2000).

Previous investigations suggest that increased uracil-misincorporation after TS inhibition may result in enhanced toxicity to TS-directed chemotherapy. In this study, we investigate the relative impact of inhibition of human dUTPase expression on dUTP accumulation and chemosensitivity to TS inhibition in an effort to further validate the role of dUTPase as a determinant of drug resistance and as a target for therapeutic development (Ladner, 2001). To address these issues, we performed siRNA-mediated knockdown of dUTPase in human cancer cell lines to assess the impact of lowered dUTPase expression on cell lines that do or do not inherently accumulate dUTP after inhibition of TS by FUdR.

Materials and Methods

Cell Culture. The human cancer cell lines SW620, MCF-7, and HT29 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in 5% CO₂ at 37°C in a humidified environment. These cells were propagated in Liebovitz's L-15 medium, modified Eagle's medium, and McCoy's 5A media, respectively, and supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 mM pyruvate. MEM was further supplemented with 10 µg/ml bovine insulin.

RNA Oligonucleotides. siRNA oligonucleotides purchased from QIAGEN (Valencia, CA) were designed according to the manufacturer's guidelines to target a portion of the human dUTPase open reading frame common to both nuclear and mitochondrial isoforms [siDUT/230 (sense, 5'-CGGACAUUCAGAUAGCGCUGd(TT); and antisense, 5'-AGCGCUUAUCUGAAUGUCCGd(TT)]. siRNA oligonucleotides targeting the green fluorescent protein (siGFP-22) were also purchased from QIAGEN for use as a negative control. Double-stranded siRNAs were generated by mixing the corresponding pair of sense and antisense oligonucleotides in Suspension Buffer (100 mM potassium acetate, 30 mM HEPES-KOH, and 2 mM magnesium acetate, pH 7.4) to obtain a 20 µM solution. The reaction mixture was heated to 95°C for 1 min and then incubated at 37°C for 1 h, aliquoted, and stored at -20°C.

Transfection of RNA Oligonucleotides. Approximately 6 × 10⁶ cells were plated per 150 × 25 mm circular tissue culture dish in media containing 10% fetal bovine serum to give ~50% confluence. Transfection of RNA oligonucleotides was performed using the Oligofectamine Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations, with a final oligonucleotide concentration of 200 nM. After 24 h, cells were trypsinized, counted, and seeded into the appropriate dishes to ensure that cells used for all

subsequent experiments had the same transfection efficiency. All experiments were performed in triplicate.

Western Blot Analysis. Monolayer cultures were rinsed with PBS, and harvested in PBS containing a 1:1000 dilution of the Protease Inhibitor Cocktail Set III (100 mM AEBSF, HCl, 80 µM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin hemisulfate, and 1 mM pepstatin A) purchased from Calbiochem (La Jolla, CA). Cells were lysed by sonication and protein concentration was determined using the Bio-Rad (Hercules, CA) protein assay system as directed. Cell extracts (40 µg) were subjected to 15% SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis was performed as described previously (Ladner and Caradonna, 1997). Blots were probed with the following antibodies: affinity-purified rabbit anti-dUTPase (1:500) and rabbit anti-GAPDH (1:1000) (Ladner et al., 1996).

Growth Inhibition Assay. At 24 h after transfection, cells were trypsinized and seeded at 3 × 10³ cells/well in 96-well tissue culture plates. Cells were treated continuously with FUdR or paclitaxel (Taxol) at serial 10-fold dilutions beginning at 48 h after transfection. After a 72-h incubation, 20 µl of CellTiter 96 Aqueous One Solution (Promega, Madison, WI) was added to each well, then incubated at 37°C for a ~4-h period. Triplicate plates were analyzed on a Spectra Max 190 micro-plate reader (Amersham Biosciences, Piscataway, NJ) at A₄₉₀, and the IC₅₀ values for each cell line were calculated from sigmoidal-dose-response curve fits of the data using the Prism statistical software (GraphPad Software, San Diego, CA). Results are representative of three independent experiments.

Clonogenicity Assay. Transfected cells were treated for 24 and 48 h with FUdR (100 or 1000 nM) or paclitaxel (50 nM) then washed in PBS, trypsinized, counted, and seeded at a density of 2000 cells/well in the first well of a six-well tissue culture dish. Two-fold serial dilutions were performed in the remaining five wells, and cells were incubated for approximately 2 weeks to allow colony formation. Colonies were then stained with 1% crystal violet (Sigma, St. Louis, MO), and those colonies possessing >50 cells were counted. All experiments were performed in triplicate.

Nucleotide Pool Determination. dNTP pool sizes were determined by an enzymatic method as described previously (Sherman and Fyfe, 1989). We have further modified this protocol to detect TTP and dUTP pools independently by preincubating cellular extracts with and without recombinant dUTPase (Horowitz et al., 1997). Cells (1.5 × 10⁶) treated with 0, 100, or 1000 nM FUdR for 24 h were harvested by centrifugation, washed, and resuspended in 100 µl of 10% TCA and processed as described. To measure TTP and dUTP pools independently, neutralized cell extracts were treated with and without dUTPase (50 ng of recombinant protein/reaction) for 1 h at 37°C in a reaction mix that contained 50 mM Tris-Cl, pH 8.0, 10 mM MgCl₂, and 50 mM NaCl. For TTP and dUTP analysis, assay mixes contained 2.5 µM [³H]dATP (18 Ci/mmol) (Amersham Biosciences), and 25 µM TTP/dUTP-specific oligonucleotide primer-templates. Quantitative values for nucleotide concentration were derived from a standard curve that was performed with each determination. All determinations were performed in triplicate.

Pulsed Field Gel Electrophoresis. SW620, MCF-7, and HT29 cells (1.5 × 10⁶) treated with 0, 100, or 1000 nM FUdR for 48 h were harvested, washed in PBS, and resuspended in 500 µl of 10 mM Tris, pH 7.2, 20 mM NaCl, and 50 mM EDTA. The cell suspension was then mixed with 500 µl of 2% pulsed-field certified agarose (Bio-Rad) that was premelted and equilibrated to 50°C and quickly transferred to plug molds. After solidification at 4°C for 15 min, the agarose plugs were incubated for 16 h at 50°C in proteinase K reaction buffer [100 mM EDTA, pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, and 1 mg/ml proteinase K] in a volume equivalent to 1 ml of buffer for each ml of agarose plug. After incubation, plugs were washed four times in 50 ml of wash buffer (20 mM Tris, pH 8.0, and 50 mM EDTA) for 1 h at room temperature with gentle agitation. Pulsed field electrophoresis was carried out on a CHEF-DR III apparatus (Bio-Rad). Blocks containing 2 to 3 × 10⁵ cells were loaded

onto a 1% agarose gel, and DNA fractionation was carried out at 6 V/cm with a reorientation angle of 120°. The switching interval was ramped from 60 to 120 s over a total run time of 24 h. The running buffer, 0.5× Tris-borate/EDTA (45 mM Tris-borate, pH 8.0, and 1 mM EDTA), was recirculated at 14°C. Size standards were *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Bio-Rad), and DNA was visualized by ethidium bromide staining. Gels shown are representative of experiments performed in triplicate.

Results

Targeted siRNAs Suppress dUTPase Expression in Human Cancer Cell Lines. High levels of dUTPase have previously been shown to correlate with resistance to TS inhibitors in certain cell lines as well as in metastatic colon cancer (Canman et al., 1993; Canman et al., 1994; Parsels et al., 1998; Ladner et al., 2000; Tinkelenberg et al., 2002). Despite this association, it remains unclear to what extent dUTPase expression and the uracil misincorporation pathway contribute to the cytotoxic events resulting from TS inhibition. To further validate dUTPase as a therapeutic target, we designed a 21-nucleotide duplex siRNA directed against a portion of the coding region of human dUTPase (siDUT/230) to suppress dUTPase expression by RNAi. Two isoforms of human dUTPase have been identified that are transcribed from the same gene and are localized to the nucleus (DUT-N) and mitochondria (DUT-M) respectively (Ladner et al., 1996; Ladner and Caradonna, 1997; Tinkelenberg et al., 2003). As shown in Fig. 1, the target sequence is shared between both isoforms. In addition, we used an siRNA oligonucleotide duplex directed against the green fluorescent protein (siGFP-22) for a negative control.

To perform this analysis, each siRNA duplex was transfected into two human colon cancer cell lines, HT29 and SW620, and one human breast cancer cell line, MCF-7. The colon cancer cell lines were selected based on previous studies demonstrating that SW620 cells have higher levels of dUTPase activity than HT29 cells and are less sensitive to the TS inhibitor FdUR (Canman et al., 1992, 1993, 1994). At 48 and 96 h after transfection, cells were harvested and dUTPase expression was analyzed by Western blot analysis and enzymatic activity assays. siDUT/230 induced a marked reduction in both DUT-N and DUT-M levels in SW620, MCF-7, and HT29 cell lines at 48 and 96 h after transfection (Fig. 2A). Enzymatic activity assays revealed a ~75% decrease in dUTPase activity in both SW620 and MCF-7 cells and a ~50% decrease in HT29 cells at 48 h after transfection (Fig. 2B). The siGFP-22 control had no effect on dUTPase

expression or activity (Fig. 2), demonstrating that the inhibitory effects of siDUT/230 are specific for this oligonucleotide duplex. In addition, the siDUT/230 oligonucleotides did not cause a nonspecific effect on gene expression, as demonstrated by the stability of TS expression at 48 or 96 h after transfection (data not shown). These data indicate that siDUT/230 can effectively suppress dUTPase expression in human cancer cell lines by RNAi.

Knockdown of dUTPase Expression Alters the Nucleotide Pool Profile of TS-Inhibited Cells. To investigate the effects of dUTPase suppression on nucleotide pool levels, we quantitated cellular TTP and dUTP pools after transfection with siDUT/230 and siGFP-22. In non-drug-treated SW620 cells, transfection with siDUT/230 did not induce significant changes in TTP and dUTP pools compared with cells transfected with siGFP-22. To inhibit TS, we used FdUR, which is readily converted to the active metabolite FdUMP yet avoids the potential RNA effects that may be introduced by using 5-FU (Canman et al., 1992; Longley et al., 2003). After a 24-h treatment with 1 μ M FdUR, SW620 cells transfected with the siGFP22 control before drug addition displayed a nearly complete depletion of TTP pools demonstrating the successful inhibition of TS (Fig. 3A). There was no evidence of dUTP pool expansion, suggesting that the levels of dUTPase in SW620 cells are sufficient to prevent dUTP accumulation during TS inhibition. In contrast, transfection with siDUT/230 before drug treatment induced the depletion of TTP and a dramatic accumulation of dUTP in response to TS inhibition. These results demonstrate that although the inhibitory effects of siDUT/230 did not elicit a significant change in TTP and dUTP pools under normal conditions, suppression of dUTPase abrogated the ability of SW620 cells to eliminate dUTP from the DNA biosynthetic pathway after treatment with FdUR.

Before drug treatment, results obtained from MCF-7 and HT29 cells were comparable with those observed in SW620 cells (Fig. 3B). TTP and dUTP pool levels in both cell lines transfected with siDUT/230 were nearly identical to those transfected with the siGFP22 control. However, results diverge between the cell lines upon FdUR treatment. Unlike SW620 cells, MCF-7 and HT29 cells transfected with siGFP-22 inherently accumulate dUTP when TS is inhibited. These data are consistent with previous reports demonstrating that HT29 cells accumulate dUTP after treatment with

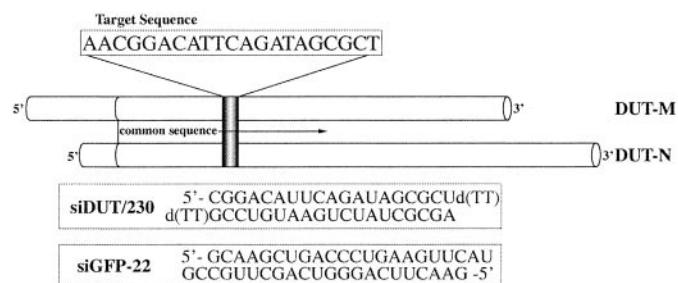


Fig. 1. Sequence of the targeted region for siRNAs of human nuclear (DUT-N) and mitochondrial (DUT-M) dUTPase. The sense (top) and the antisense (bottom) sequences of the siRNA targeting both dUTPase isoforms (siDUT/230) are shown. The sequence of the control siRNA (siGFP-22) is also shown.

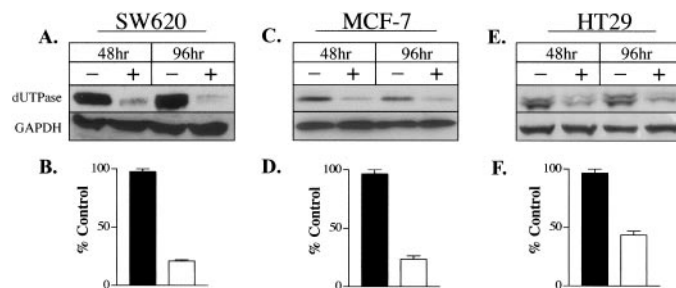


Fig. 2. Inhibition of dUTPase expression and activity after transfection with siRNA. Western blot analysis of SW620 (A), MCF-7 (C), and HT29 (E) cells treated with siGFP-22 (–) or siDUT/230 (+) for 48 and 96 h. The upper band in the dUTPase blots represents the DUT-M isoform and the lower band DUT-N (Ladner et al., 1996; Ladner and Caradonna, 1997). GAPDH was included to assess equal lane loading. B, D and E, dUTPase catalytic activities were determined after 48 h of treatment with siGFP-22 (□) or siDUT/230 (■) as described previously (Caradonna and Adamkiewicz, 1984). Bars represent S.D.

TS inhibitors (Canman et al., 1993). When MCF-7 and HT29 cells are transfected with siDUT/230 before drug treatment, there are ~2- and ~3-fold increases in dUTP levels, respectively, compared with the drug-treated siGFP-22 control. Although the suppression of dUTPase activity is not required for these cells to accumulate dUTP, it does increase the magnitude of accumulation.

Lowered Levels of dUTPase Significantly Sensitize SW620 and MCF-7 Cells to TS Inhibition. To evaluate the impact of varying degrees of dUTP accumulation on thymineless death, we determined the sensitivity of SW620, MCF-7, and HT29 cells transfected with either siDUT/230 or the siGFP-22 control to the TS inhibitor, FUDR, by both growth inhibition and clonogenicity analyses. The specificity of the effects of siDUT/230 to TS inhibition was assessed by determining the sensitivity of the cells to the non-TS-targeted drug, paclitaxel, which inhibits cell growth by binding to dynamic microtubules (Dumontet and Sikic, 1999). The growth profiles of dUTPase-inhibited and uninhibited cells were analyzed after treatment with a range of concentrations of either FUDR or paclitaxel for 72 h by growth inhibition assays performed with the CellTiter 96 Aqueous One Solution. When dUTPase was suppressed in SW620 and MCF-7 cells, the FUDR IC_{50}^{72h} concentrations decreased substantially compared with the values obtained for the siGFP-22 transfected control cells (Table 1). The IC_{50}^{72h} decreased by more than 75-fold in SW620 cells (5.5 μ M for siGFP-22 compared with 70 nM for siDUT/230,) and ~6-fold in MCF-7 cells (384 nM for siGFP-22 compared with 61 nM for siDUT/230; Table 1). These results suggest that suppression of dUTPase in these cell lines significantly increased sensitivity to the cytotoxic effects of TS inhibition. When these cells were treated with paclitaxel, however, there was virtually no change in chemosensitivity, indicating that results observed after treatment with FUDR were specific for the TS inhibitor (Table 1). In contrast, when siRNA transfected HT29 cells were treated with either FUDR or paclitaxel, there were no significant differences in the IC_{50}^{72h} concentrations; inhibiting dUTPase expression had no impact on the cellular sensitivity to these chemotherapeutic agents in this cell line (Table 1).

To determine whether alterations in sensitivity to FUDR observed by growth inhibition assays translate to changes in

the ability of cells to recover from drug treatment, we performed clonogenicity analyses. In SW620 and MCF-7 cells, there was a correlation between the suppression of dUTPase activity and decreased number of colonies formed in the presence of 100 or 1000 nM FUDR. In SW620 cells treated with 1000 nM FUDR, there was a ~60% reduction in colony formation at 24 h and ~75% reduction at 48 h of drug exposure when cells transfected with siDUT/230 are compared with those transfected with the siGFP-22 control (Fig. 4A). MCF-7 cells transfected with siDUT/230 displayed an ~40% reduction in colony formation at 24 h and an ~75% reduction after 48 h of treatment with 100 nM FUDR (Fig. 4B). Transfection of either siDUT/230 or siGFP-22 into both SW620 and MCF-7 cells produced no changes in cell survival in the presence of paclitaxel (data not shown). These results indicate that the observed effect on survival after FUDR treatment was not caused by a nonspecific decrease in colony formation; rather, it was limited to sensitivity to the inhibition of TS. These observations were not seen in HT29 cells, however, which displayed no loss of clonogenicity after treatment with either 100 nM FUDR or 50 nM paclitaxel when dUTPase was suppressed (Fig. 4C).

Induction of dUTP Pool Accumulation in SW620 and MCF-7 Cells Induces Enhanced DNA Damage. Previous studies have demonstrated that variations in FUDR-induced DNA damage exist between different cell lines that are likely to be caused by differences in the events after TS inhibition (Canman et al., 1993). To determine whether the increased sensitivity of SW620 and MCF-7 cells transfected with siDUT/230 correlated with an increase in DNA double strand break formation, we measured DNA fragmentation by pulsed-field gel electrophoresis (PFGE). After treatment with either 100 or 1000 nM FUDR for 48 h, SW620 cells with compromised dUTPase activity demonstrated a dose-dependent increase in DNA fragmentation as evidenced by greater DNA migration into the gel compared with the siGFP-22-transfected control (Fig. 5). These data indicate that the inherent resistance of SW620 cells to DNA damage after FUDR treatment may be partially overcome by the inhibition of dUTPase activity and the resultant expansion of dUTP pools. Although MCF-7 control cells incur a substantial amount of DNA fragmentation after treatment with FUDR, the suppression of dUTPase and the enhanced expansion of

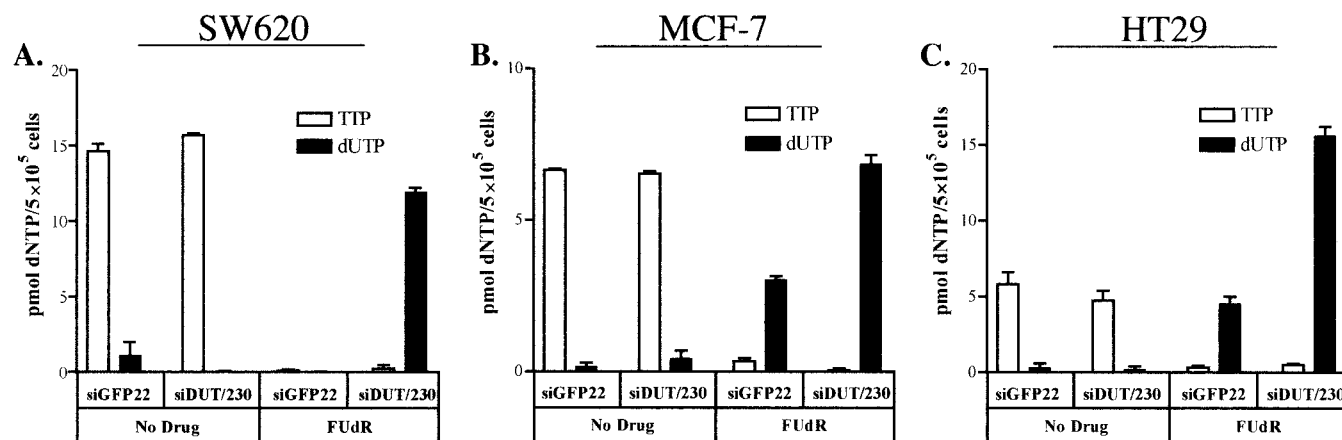


Fig. 3. Nucleotide pool analysis of FUDR-treated cells. Both TTP (□) and dUTP (■) pool levels were determined in SW620 (A), MCF-7 (B), and HT29 cells (C) that were first transfected with either siGFP-22 or siDUT/230 and then treated for 24 h with 1 μ M FUDR or were left untreated. Cells were subsequently harvested and processed as described under *Materials and Methods*. Bars represent S.D.

dUTP pools was correlated with a significant increase in the magnitude of damage that occurs after the inhibition of TS (Fig. 5). HT29 cells, in contrast, exhibited no significant difference in the degree of DNA fragmentation between dUTPase depleted and control cells, suggesting that, in this cell line, inhibition of dUTPase does not increase DNA double strand break formation despite a 3-fold increase in dUTP accumulation.

Discussion

A considerable body of evidence exists implicating extensive uracil misincorporation and its associated misrepair as contributing mechanisms of cytotoxicity after TS inhibition (Aherne and Browne, 1999; Ladner, 2001). It has been demonstrated that overexpression of the enzyme dUTPase counters dUTP pool expansion and may protect certain cell lines from the resultant DNA damage and toxicity (Curtin et al., 1991; Canman et al., 1993; Canman et al., 1994). Despite this evidence, the potential of dUTPase and the uracil misincorporation pathway as exploitable therapeutic targets has yet to be clearly validated.

We approached this issue by designing siRNA directed against dUTPase to determine how a decrease in the expression of this gene alters sensitivity of SW620, MCF-7, and

HT29 human cancer cell lines to the TS inhibitor FUDR. Previous studies have demonstrated that SW620 colon cancer cells are significantly resistant to the effects of FUDR treatment compared with HT29 cells, despite the fact that they are equally sensitive to TS inhibition (Canman et al., 1993). It was hypothesized that variability in cellular resistance to FUDR may be partially attributed to differences in dUTP pool expansion. Herein, we demonstrated directly that levels of dUTPase activity govern the cellular capacity to accumulate dUTP pools and can dramatically affect chemosensitivity to TS inhibition in human cancer cells.

In both SW620 and MCF-7 cells, we achieved a ~75% decrease of dUTPase activity after transfection with siDUT/230. Although dUTPase is an essential enzyme, suppression by RNAi alone had no measurable effect on cellular viability, growth rate, or dUTP pools, suggesting that the residual enzymatic activity was sufficient for the elimination of dUTP under normal cellular conditions. When SW620 cells transfected with siDUT/230 were treated with FUDR, there was a dramatic increase in the dUTP/dTTP ratio. This was accompanied by increased DNA double strand break formation and greatly enhanced chemosensitivity. These data demonstrate for the first time the ability to overcome an innate cellular resistance to dUTP pool accumulation after treatment with FUDR. In this scenario, the enabled uracil misincorporation pathway significantly induced drug sensitivity in an otherwise FUDR-resistant cell line, suggesting that the suppression of dUTPase activity can directly synergize with TS inhibition by increasing the cellular vulnerability to dUTP pool expansion. Further evidence supporting this hypothesis was observed in MCF-7 cells, where depletion of dUTPase correlated with increased dUTP pool expansion, greater DNA double strand break formation, and enhanced chemosensitivity. Together, these results further validate dUTPase as a promising target for drug development (McIntosh and Haynes, 1997; Grasser et al., 2001; Ladner, 2001), whose partial inhibition may overcome an important mechanism of drug resistance that is inherent to the widely used anticancer agents that inhibit thymidylate metabolism. It is important to note that because these results were achieved without the complete loss of dUTPase activity, a meaningful inhibition by a small molecule antagonist should be pharmacologically feasible.

TABLE 1

Summary of growth inhibition assays for siRNA-transfected cell lines SW620, MCF-7, and HT29 cells transfected with either siGFP-22 or siDUT/230 were assayed for growth inhibition using CellTiter 96 Aqueous One Solution after treatment for 72 h with FUDR or paclitaxel. Data are presented as the mean \pm S.D. of three experiments carried out in triplicate.

	IC ₅₀	
	FUDR (72 h)	Paclitaxel (72 h)
	μM	nM
SW620		
siGFP-22	5.50 \pm 2.00	2.4 \pm 2.0
siDUT/230	0.07 \pm 0.05 ^a	2.3 \pm 2.0
MCF-7		
siGFP-22	0.384 \pm 0.050	1.0 \pm 0.8
siDUT/230	0.061 \pm 0.030 ^a	1.3 \pm 0.7
HT29		
siGFP-22	0.0213 \pm 0.010	1.9 \pm 1.0
siDUT/230	0.0102 \pm 0.006	1.7 \pm 1.0

^a Significant sensitivity compared with the siGFP-22 transfected control.

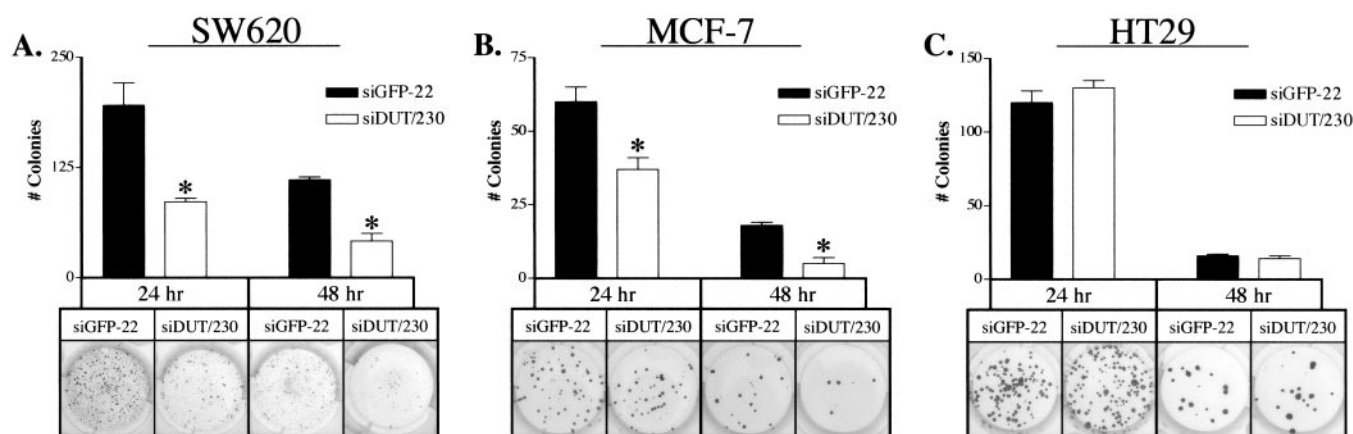


Fig. 4. Clonogenicity analysis of siRNA-transfected cells treated with FUDR. Cells were treated for either 24 or 48 h with 100 or 1000 nM FUDR. Illustrated are SW620 cells treated with 1000 nM FUDR (A) and MCF-7 (B) and HT29 (C) cells treated with 100 nM FUDR. Results are representative of three independent experiments. *, $p < 0.001$, Student's t test. Bars represent S.D.

Unlike SW620 and MCF-7 cells, HT29 cells transfected with siDUT/230 did not display an increased sensitivity to FUDR despite a 50% reduction in dUTPase activity. Like MCF-7 cells, HT29 cells, which have lower levels of dUTPase than SW620 cells, intrinsically accumulate dUTP and undergo DNA double strand breaks after TS inhibition (Canman et al., 1993). Although there was a 3-fold increase in dUTP pools when dUTPase was suppressed, there was no apparent increase in DNA damage. These data suggest that the effects of uracil misincorporation may reach a saturation point, and that additional dUTP pool expansion beyond this threshold level has no significant additive effect on toxicity resulting from TS inhibition in this cell line.

The potential of dUTPase as a target for drug development is supported by previous studies that reported broad variations in enzyme activity between tumor types: if differences in dUTPase levels did not exist, this enzyme would be an unlikely critical determinant of drug sensitivity to TS inhibitors in vivo (Fleischmann et al., 1999; Ladner et al., 2000). In addition, preliminary studies performed in our lab demonstrate that dUTPase expression in tumor specimens obtained from patients with metastatic colon cancer varies both in quantity and intracellular localization (Ladner et al., 2000). Of 20 tumor samples analyzed, 40% had elevated dUTPase expression in the nucleus of the cancer cells. It is noteworthy that elevated nuclear dUTPase expression was shown to be significantly associated with poorer response and decreased time to tumor progression after treatment with 5-FU/leucovorin-based chemotherapy (Ladner et al., 2000). It is conceivable that, if the tumors with elevated dUTPase expression respond in a manner similar to that of SW620 and MCF-7 cells, partial inhibition of dUTPase may access the benefit of the uracil misincorporation pathway, potentially overcoming

inherent drug resistance. If this hypothesis is true, development of an effective inhibitor of dUTPase may significantly enhance the efficacy of TS inhibitors currently used in the clinic.

Although these results highlight the pivotal role of dUTPase in mediating chemosensitivity, several other components of the uracil misincorporation pathway must be considered potential determinants of toxicity. It has been postulated that uracil-base excision repair is responsible for much of the DNA fragmentation observed in cells that accumulate dUTP after drug treatment (Hochhauser and Weiss, 1978; Goulian et al., 1980b; Sedwick et al., 1981; Ingraham et al., 1982; Goulian et al., 1986). Variation in enzymes such as uracil-DNA glycosylase and the single-stranded monofunctional uracil-DNA glycosylase (hSMUG1), which initiate this repair process in human cells, may affect the extent of DNA damage incurred and, therefore, cytotoxicity (Caradonna and Muller-Weeks, 2001; Tinkelenberg et al., 2002; Elateri et al., 2003). In addition, there are other enzymes that may contribute to the accumulation of dUTP pools such as dCMP deaminase, whose activity results in the deamination of dCMP to dUMP, and ribonucleotide reductase, which converts UDP to dUDP (Aherne and Browne, 1999). Variation in the expression or regulation of these enzymes may enhance the cellular capacity to accumulate dUTP and potentially overwhelm dUTPase. To fully exploit thymidylate metabolism as a therapeutic target, there needs to be a comprehensive understanding of all relevant enzymes within this pathway. With this knowledge, it may be possible to better predict therapeutic response and enhance clinical efficacy TS-directed chemotherapy by alleviating chemoresistance.

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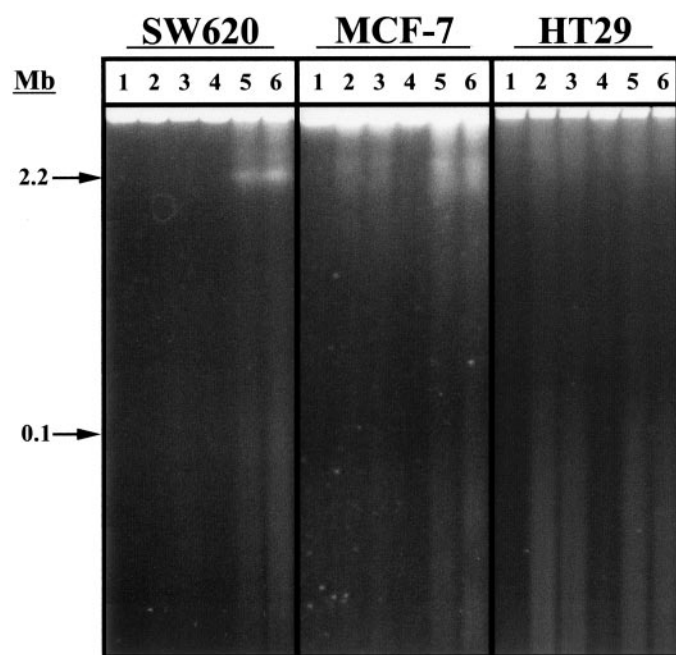


Fig. 5. Double strand break formation in siRNA-transfected cells treated with FUDR. SW620, MCF-7, and HT29 cells were transfected with either siGFP-22 (lanes 1–3) and or siDUT/230 (lanes 4–6); after 24 h, the cells were seeded for PFGE analysis of double strand break formation. Cells were exposed to 0 (lanes 1 and 4), 100 nM (lanes 2 and 5), or 1 μ M (lanes 3 and 6) of FUDR for 48 h and then harvested and analyzed by PFGE as described under *Materials and Methods*.

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