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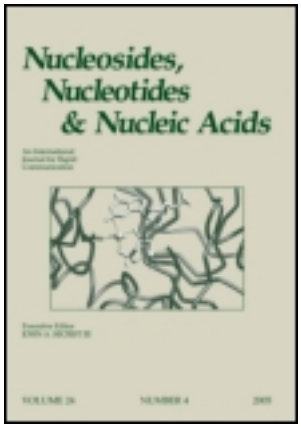

Nucleosides, Nucleotides and Nucleic Acids

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CELLULAR RESPONSE TO EFFICIENT dUTPase RNAi SILENCING IN STABLE HeLa CELL LINES PERTURBS EXPRESSION LEVELS OF GENES INVOLVED IN THYMIDYLATE METABOLISM

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□ *dUTPase is involved in preserving DNA integrity in cells. We report an efficient dUTPase silencing by RNAi-based system in stable human cell line. Repression of dUTPase induced specific expression level increments for thymidylate kinase and thymidine kinase, and also an increased sensitization to 5-fluoro-2′-deoxyuridine and 5-fluoro-uracil. The catalytic mechanism of dUTPase was investigated for 5-fluoro-dUTP. The 5F-substitution on the uracil ring of the substrate did not change the kinetic mechanism of dUTP hydrolysis by dUTPase. Results indicate that RNAi silencing of dUTPase induces a complex cellular response wherein sensitivity towards fluoropyrimidines and gene expression levels of related enzymes are both modulated.*

Keywords 5-fluoro-uracil; 5-fluoro-2′-deoxyuridine; dUTPase; thymidylate metabolism; RNAi

INTRODUCTION

Preservation of genome integrity is crucial for cellular viability and is provided for by DNA damage repair mechanisms. Fine-tuned regulation of the levels of the DNA building block nucleotides dTTP and dUTP contributes to DNA damage repair by preventing incorporation of dUMP moieties via DNA polymerases. The enzyme dUTPase, which plays an important role in the de

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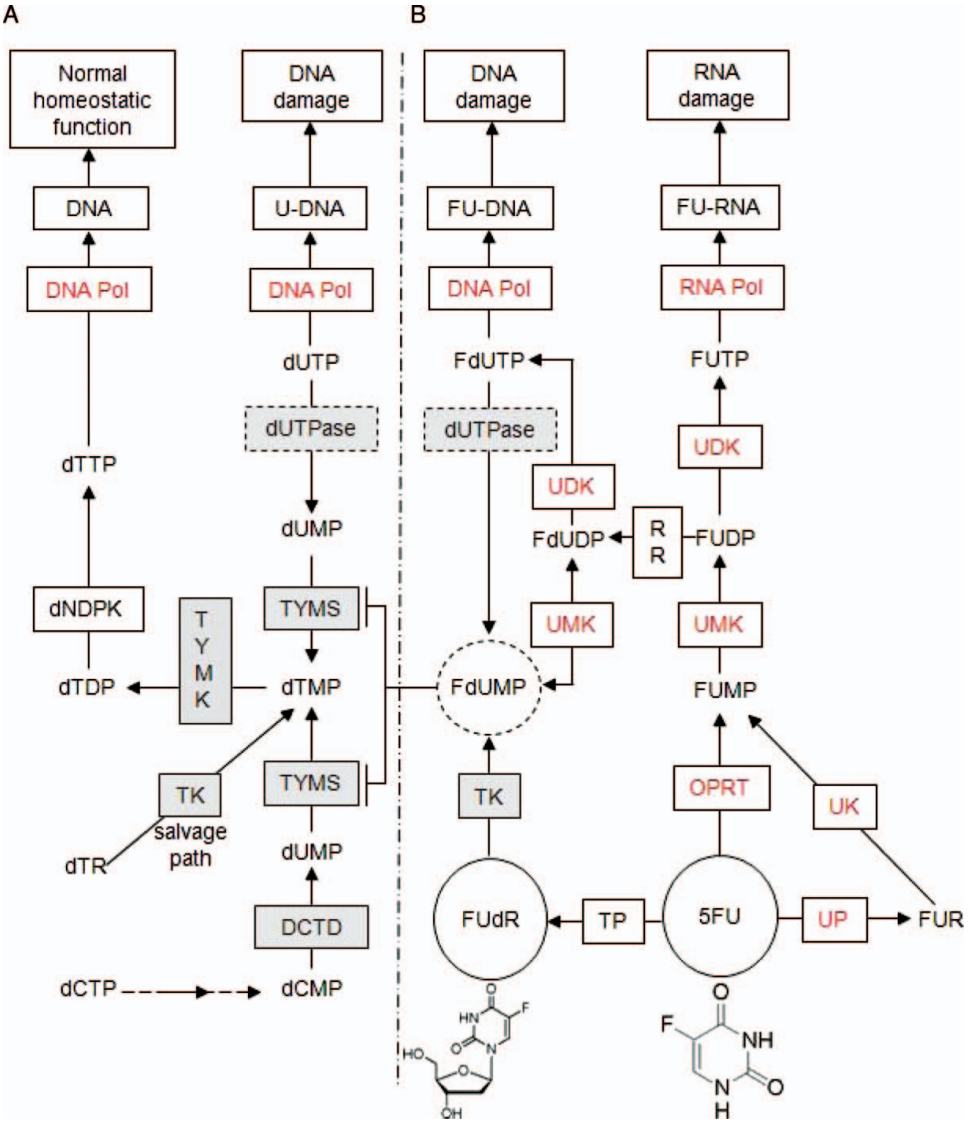


FIGURE 1 Pathways of deoxythymidylate biosynthesis and the metabolism of fluoropyrimidines (5FU and FUdR) in human cells. A) In the de novo deoxythymidylate synthetic pathway, thymidylate synthase (TYMS) processes the rate-limiting step of converting deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). In human cells, there are two main sources to provide dUMP as a substrate for TYMS. In one of the pathways, deoxyuridine triphosphatase (dUTPase) catalyzes the conversion of deoxyuridine triphosphate (dUTP) into dUMP. In the other pathway, deoxycytidine deaminase (DCTD) catalyzes deamination of deoxycytidine monophosphate (dCMP) to dUMP. Alternatively, in the salvage pathway, thymidine kinase (TK) produces dTMP by phosphorylation of deoxythymidine (TdR). Subsequent phosphorylation of dTMP by thymidylate kinase (TYMK) results deoxythymidine diphosphate (dTDP), which is converted to deoxythymidine triphosphate (dTTP) in the further phosphorylation step catalyzed by deoxynucleotide diphosphate kinase (dNDPK). dTTP is used for DNA synthesis in the normal homeostatic cell function. dUTPase activity is essential for DNA integrity, because it prevents dUTP misincorporation into DNA during replication and repair. B) After cellular uptake of 5-fluorouracil (5FU), it could be converted into several active metabolites.

novo dTTP biosynthesis pathway, is among the major factors responsible for this regulation: the enzyme-catalyzed reaction sanitizes the dNTP pool by removing dUTP and producing dUMP, the precursor for dTTP biosynthesis.[1] Lack of dUTPase leads to expansion of the dUTP pool and high levels of uracil-substitutions in DNA, which overloads the capacity of uracil-directed base-excision repair.[2–5] In the absence of dUTPase function, repair synthesis will reincorporate uracil and the repair pathway is therefore transformed into a hyperactive futile cycle, resulting in multiple DNA breaks and cell death. This process was termed thymine-less cell death.[6,7] The pathway of thymidylate biosynthesis consists of both de novo and salvage constituents. Besides dUTPase, deoxycytidine deaminase (DCTD) is considered to supply precursors for the de novo biosynthesis. In the case of salvage pathway thymidine kinase (TK) plays an important role in formation thymidine to dTMP, which is converted to dTDP in a subsequent phosphorylation step catalyzed by thymidylate kinase (TYMK). TYMK is an essential enzyme which has an integration function on both constituents of the dTTP biosynthesis. Finally, the dTDP will be the substrate for deoxynucleotide diphosphate kinase (dNDPK), providing dTTP nucleotides for DNA synthesis[1] (Figure 1A).

Thymine-less cell death is also a widely used strategy in anticancer chemotherapy applying fluoropyrimidines.[8] 5-Fluorouracil (5FU) and 5-fluoro-2′-deoxyuridine (FUdR) elicit cell killing by a complex mechanism of action (cf. Figure 1B). One of the active metabolites formed from 5FU within the cells is 5-fluoro-2′-deoxyuridine monophosphate (FdUMP), which binds to and inhibits thymidylate synthase (TYMS).[8] TYMS processes the rate-limiting step of converting dUMP to dTMP. Further active metabolites are the 5-fluoro-uridine triphosphate (FUTP) and 5-fluoro-2′-deoxyuridine triphosphate (FdUTP), which may be incorporated into RNA and DNA,

FIGURE 1 (Continued) During the first step of the main anabolic pathway, 5FU is converted by orotate phosphoribosyl transferase (OPRT) into fluorouridine monophosphate (FUMP). Alternatively, FUMP can also be produced through the 5-fluoro-uridine (FUR) intermediate by the sequential actions of uridine phosphorylase (UP) and uridine kinase (UK). Then FUMP is phosphorylated by uridine monophosphate kinase (UMK), resulting in fluorouridine diphosphate (FUDP), which is either reduced by ribonucleotide reductase (RR) to fluorodeoxyuridine diphosphate (FdUDP) and subsequently converted to fluorodeoxyuridine triphosphate (FdUTP) by uridine diphosphate kinase (UDK), or further phosphorylated by UDK to fluorouridine triphosphate (FUTP). FUTP and FdUTP represent two major active metabolites of 5FU. FUTP could be misincorporated into RNA by RNA polymerase (RNA Pol) resulting RNA damage by perturbation and disruption the normal RNA processing. FdUTP could be either hydrolyzed to pyrophosphate and fluorodeoxyuridine monophosphate (FdUMP) by dUTPase or misincorporated into DNA by DNA polymerase (DNA Pol) during the replication process, causing DNA damage. During the second relevant anabolic pathway, 5FU is converted by thymidine phosphorylase (TP) to fluorodeoxyuridine (FUdR) and subsequently phosphorylated by TK, producing the other active metabolite, FdUMP, which is a potent inhibitor of TYMS. FdUMP can be produced from FdUDP in the reversible reaction of UMK as well. FdUMP can bind to the nucleotide-binding site of TYMS, resulting a ternary complex with the enzyme and its co-factor methylene tetrahydrofolate (CH_2-THF) (not shown).[30] Blocking of TYMS activity by FdUMP is a rate limiting step of the dTTP synthesis.

respectively.[9–13] FUTP misincorporation into RNA is considered to induce major disruption of RNA processing pathways including pre-rRNA and pre-mRNA maturing, posttranscriptional modification of tRNA and proper functioning of snRNA/protein complexes affecting gene splicing. Furthermore, previous studies revealed the fact that polyadenylation of mRNA is strongly inhibited at a low concentration of 5FU.[12,14,15] For a detailed understanding of the mechanism of action of fluoropyrimidines, several studies focused on different enzymes in the thymidylate synthesis pathway.[8,13] Interestingly, resistance against 5FU was shown to be developed as a result of dUTPase overexpression in different cancer cell lines.[16,17]

With respect to the relative importance of enzymes taking part in the dTTP biosynthesis pathway, some approaches were performed to repress the dUTPase gene (DUT), TYMK and TYMS genes in different types of human cell lines. Transient silencing of dUTPase by RNAi and its effect on sensitizing cells against FUdR, but not 5FU, usually administered in the clinics, was addressed.[5] These experiments resulted in significant transient silencing levels, with varied efficiency in different cell lines.[5,18,19] Efficient RNAi experiments were also reported to silence the TYMK and TYMS genes in colon cancer cell lines.[20]

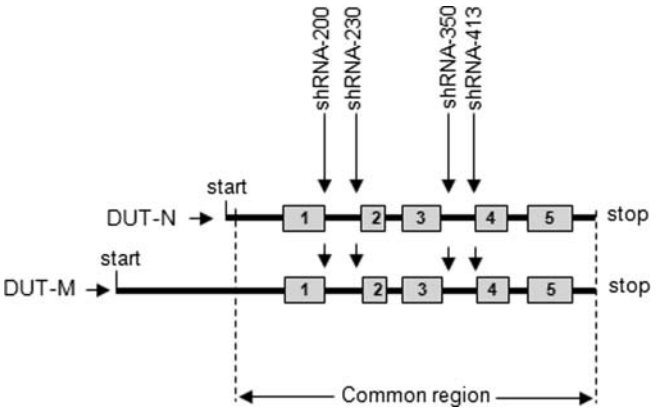
In the present study, we aimed at generation of stable HeLa cell lines in which either the DUT or the TYMK gene is constantly silenced by a vector-based RNAi system. With respect to the essential functions of the aforementioned enzymes in thymidylate metabolism, we aimed to characterize the gene expression pattern of DUT, TK, TYMK, DCTD, and TYMS enzymes as molecular responses to the gene silencing (cf. Figure 1A). Cell viability experiments of the generated stable cell lines with respect to the effect of fluoropyrimidines (5FU, FUdR) were also performed. Additionally, in enzyme kinetics and ligand binding experiments, we addressed the catalytic mechanism of human dUTPase in the reaction of 5-fluoro-2′-deoxyuridine triphosphate (FdUTP) cleavage.

RESULTS AND DISCUSSION

Effective Silencing of the DUT Gene by Vector-Based RNAi

Our main goal was to examine the cellular and molecular events caused by DUT gene silencing in human TR-HeLa cell line using a vector-based RNAi system.

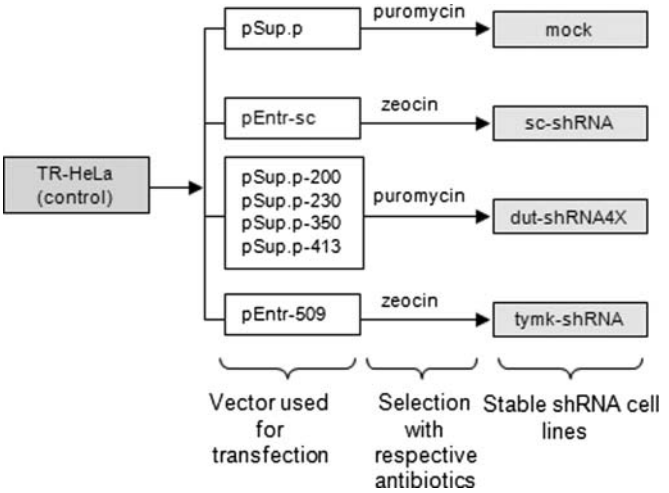
To achieve an increased RNAi efficiency, we used four shRNA oligos (Table 1) with distinct recognition sites on the common coding regions of the mRNAs of both dUTPase isoforms (Scheme 1). As a negative control, we used a scrambled-shRNA (sc-shRNA) construct (Table 1). Stable transfected



SCHEME 1 Recognition sites for shRNA oligos directed to mRNA of both dUTPase isoforms. Schematic representation shows the coding sequences of dUTPase isoforms (DUT-N, nuclear isoform, DUT-M, mitochondrial isoform, respectively) and the target positions of shRNA oligos directed to distinct sequences (indicated with arrows) placed in the common coding region of DUT mRNAs. The five conserved motifs are boxed in gray. The sequence of the shRNA oligos are given in Table 1.

cell lines were generated according to methods described (Scheme 2) to create a highly efficient silencing system.

Immunoblot results (Figure 2A) revealed that no protein could be observed in the case of the dut-shRNA4X cell line compared to control cell lines (mock and untransfected control), indicating an efficient silencing effect of the shRNA constructs. Quantitation of mRNA levels of cell lines correlated positively to protein levels detected by immunoblot (Figure 2B,



SCHEME 2 Generation of stable cell lines used in the present study. TR-HeLa cells were transfected with the respective vector-constructs (indicated in boxes with white background) and further selected by using the appropriate antibiotics to generate stable cell lines (indicated in boxes with gray background). Experiments were conducted as described in the Materials and Methods section.

TABLE 1 Summary of the shRNA vector constructs used in RNAi experiments

Name of shRNA vector constructs	Target sites	
	Location in coding sequence	Sequence (sense)
pSup.p-200	200–219	taccacctatggagaaagc
pSup.p-230	230–249	cggacattcagatagcgct
pSup.p-350	350–369	aatgttggtgttgtactgt
pSup.p-413	413–432	ttgcacagctcatttgcga
pEntr-509	509–527	agacacgactttgaactgg
pEntr-sc	—	Gaacctggtgcaacgtata

Name of the shRNA vector constructs, localization sites, and sense sequences are indicated.

cf. also Table 2). dUTPase mRNA level was significantly reduced (17-fold at $P < 0.004$) in the dut-shRNS4X cell line compared to the control cell line (sc-shRNA).

In our system, we could achieve robust RNAi silencing on the DUT gene with practically undetectable protein levels.

Effect of the DUT Gene Repression by RNAi on Expression Levels of Other Genes Involved in Thymidylate Metabolism

We wished to assess the putative effects of dUTPase silencing on expression the levels of other enzymes closely involved in thymidylate metabolism. Based on this pathway, we selected four additional enzymes (cf. shaded boxes on Figure 1A) that catalyze reactions sequential to dUTPase.

Respective mRNA levels were determined by quantitative PCR measurements in control (mock, sc-shRNA and untransfected control) and in dut-shRNA4X cell lines (Figure 2C). Effective silencing of the DUT gene led to a significant ($P < 0.02$) 3.2-fold increment of the TYMK gene expression. In addition, a significant ($P < 0.04$) 2-fold elevation was also observed for the mRNA level of the TK gene as well. Interestingly, reduction of DUT expression caused a 2-fold decrease in DCTD expression, but no noticeable up-regulation could be observed for gene expression of the TYMS enzyme.

These data suggest that in our experiments, silencing of the DUT gene induces TYMK and TK gene expression increments in TR-HeLa cells. In the salvage pathway, thymidine serves as an input for dTMP synthesis via the reaction catalyzed by TK. Elevated levels of TK and TYMK gene expressions could provide a sufficient amount of dTMP and dTDP nucleotides for dTTP synthesis in cells in which the dUTPase protein is particularly undetectable. Reduction of DCTD gene expression was unexpected as the function of DCTD is similar to dUTPase as both enzymes produce the TYMS precursor dUMP. Lack of modulation of TYMS expression by dUTPase silencing should also be considered within the context of other potential levels of regulation for this enzyme, as it is known that the TYMS protein efficiently regulates

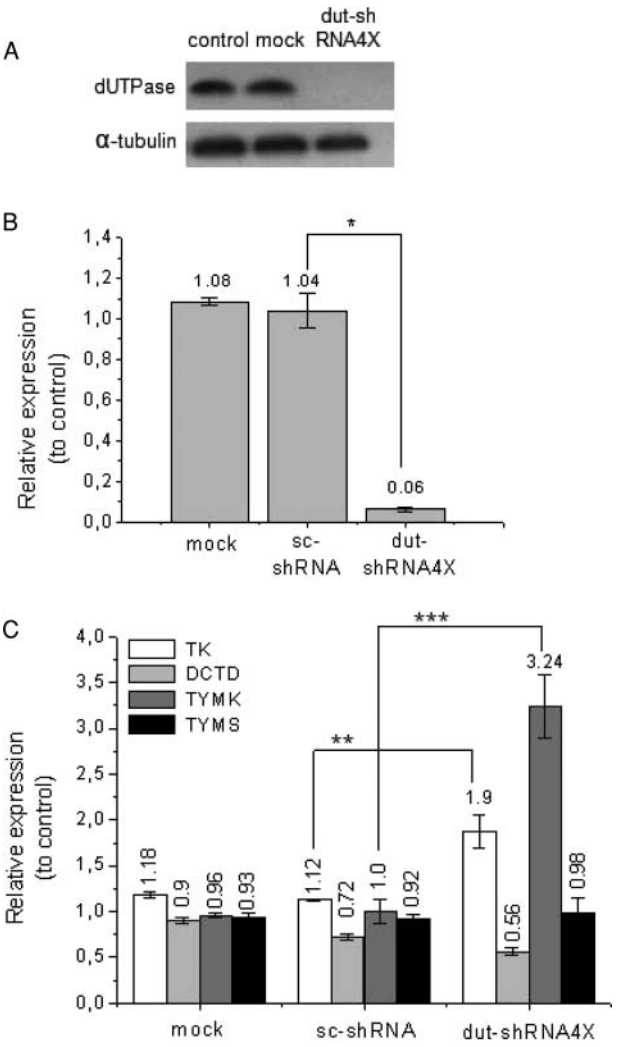


FIGURE 2 Downregulation of the DUT gene by RNAi and its effect on expression patterns of genes involved in thymidylate biosynthesis in TR-HeLa cell. A) Immunoblot assay shows the efficiency of RNAi on dUTPase protein level in dut-shRNA4X cell line compared to control cell lines (untransfected control and mock). The blots were reacted with polyclonal anti-serum to human dUTPase and with monoclonal antibody to α-tubulin, followed by reaction with a horseradish peroxidase conjugated secondary antibodies. B) Downregulation of the DUT gene detected by quantitative-PCR analysis. DUT mRNA levels were quantified in stable cell lines (mock, sc-shRNA, and dut-shRNA4X) by using specific primer pair for amplification. C) Quantitative-PCR analysis shows the effect of the DUT gene silencing on expression patterns of genes involved in thymidylate biosynthesis in dut-shRNA4X stable cell line compared to control cell lines (mock, sc-shRNA). TK, DCTD, TYMK, and TYMS genes mRNA levels were measured by using gene specific primer pairs for amplification. For immunoblot and quantitative-PCR experiments cells were treated with tetracycline at the concentration of 1 μg/ml for 72 hours, then harvested and prepared for appropriate analysis. $^{*}P < 0.004$; $^{**}P < 0.02$; $^{***}P < 0.05$. P values were calculated using a two-tailed student's t test. Data are presented as the mean $\pm$SD. of two independent relative expression ratio determinations. Experiments were conducted as described in the Materials and Methods section.

TABLE 2 Summary of gene specific primer sequences designed for quantitative-PCR analysis

Quantitative PCR primer pairs		
	Sequences (5′-3′)	
Gene	Forward	Reverse
GAPDH	gcggggctctccagaacatcatc	gacgcctgcttcaccaccttcttg
DUT	tggaagagtggctccacggt	ccgttcgcaaatgagctgtgc
TK	tccgtcgcttccagattgctcag	ctgcccctcgtcgatgcctatg
DCTD	tcgaccgatgtgaaaggctgtag	caggagcctcgcagcagttg
TYMK	tccaccagctcatgaaagacacg	tgggcagccttgggtcacttc
TYMS	ggcagatccaacacatcctccg	Caaaacacccttccagaacacacg

Primer pairs were designed by DNA STAR/Primer Select software.

its own mRNA.[21] Our present results suggest that TYMK and TK may play important roles in the reconstitution of the thymidylate biosynthesis pathway in TR-HeLa cells, in which the DUT gene is effectively repressed by RNAi.

Downregulation of dUTPase by RNAi Sensitizes Cells to Fluoropyrimidines

According to previous studies, elevated dUTPase levels protect cells against agents perturbing thymidylate de novo biosynthesis, such as fluoropyrimidines (5FU and FUdR) (16,22,23). Moderate dUTPase silencing by RNAi increased sensitivity of several cancer cell lines to FUdR,[5] but the effect was not studied in the context of 5FU agent. Comparison of the effects of 5FU and FUdR is a relevant means to assess drug-caused perturbation at RNA as compared to DNA levels, since 5FU can also result in uracil-substituted RNA, whereas FUdR is dedicated for perturbations at the DNA level (cf. Figure 1B).[24]

To determine whether the observed drastic repression of the DUT gene by RNAi had any effect on cellular response to 5FU and FUdR, dut-shRNA4X and control (sc-shRNA and untransfected control) cell lines were exposed to 5FU and FUdR in different manners. The concentration-dependent responses of cells indicated that repression of the DUT gene increases the sensitivity of dut-shRNA4X cells for both 5FU and FUdR, with a much more increased sensitization for FUdR (Figures 3A and 3B). There were large differences in IC_{50} concentration values of repressed cell line compared to values of control cell lines: the dut-shRNA4X cell line was ~6-fold more sensitive to 5FU and ~40-fold more sensitive to FUdR, relative to untransfected control (Table 3). Cell viability was examined in a time-dependent manner as well. The drug tolerances of cell lines were evaluated at fixed concentrations of 5FU and FUdR compounds. Results indicated an increased sensitivity of dut-shRNA4X cells for both 5FU and FUdR agents compared to control cells. Growth inhibition of repressed cell line was more pronounced in the case of the FUdR agent (Figures 3C and 3D).

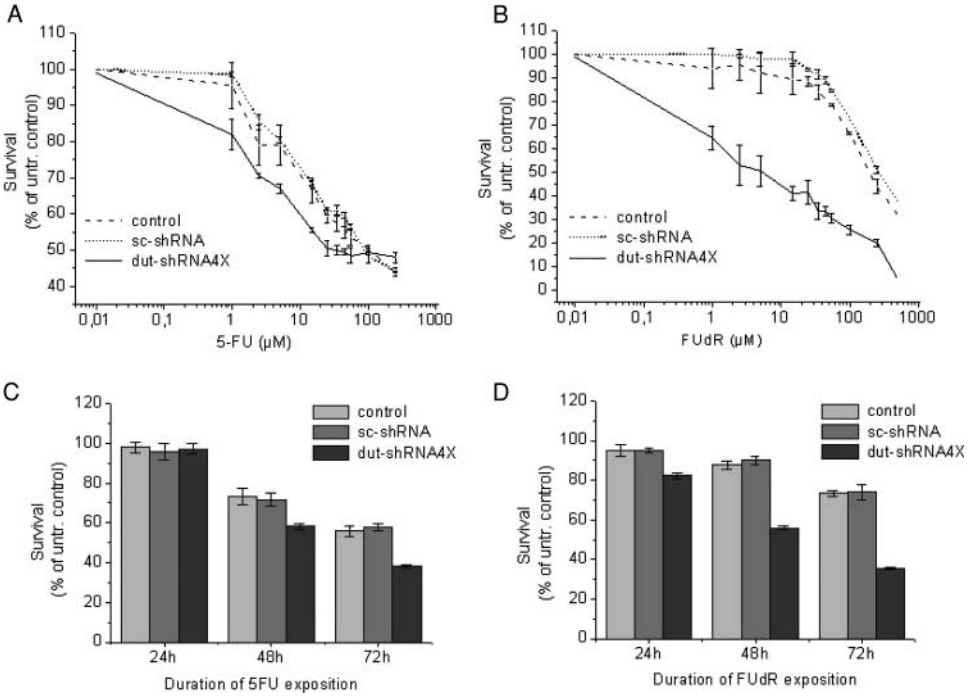


FIGURE 3 Downregulation of the DUT gene induces elevated sensitization effects to 5FU and FUdR in TR-HeLa cell. A/B) Growth inhibition effects of 5FU (A) and FUdR (B) were evaluated in concentration-dependent manner on control (– – –), sc-shRNA (. . .), and dut-shRNA4X (—) cell lines. Cells were treated for 72 hours with various concentrations of 5FU or FUdR. (*C,D*), Growth inhibition effects of 5FU (C) and FUdR (D) were carried out in time-dependent manner on control, sc-shRNA and dut-shRNA4X cell lines as well. Cells were treated with discrete concentrations of 5FU (30 μM) or FUdR (30 μM) for different time intervals (0, 24, 48, 72 hours). Inhibition assay results are reported as percentage of untreated control for each cell lines. Measurements were conducted at the present of tetracycline at the concentration of 1 μg/ml. Data represent the mean ±SD of three independent determinations at each concentration. Experiments were performed as described in the Materials and Methods section.

In summary, stable silencing of the DUT gene induces specific responses within the thymidylate metabolism pathway: a dramatic enhancement of the FUdR cell sensitizing effect is observed. DUT silencing also increases cellular sensitivity towards 5FU, the drug widely administrated in clinical chemotherapy, although in a less drastic manner. To compare the initial phase (the low concentration range) of the dose dependency curves of different drugs, it does seem that only 5FU induces an elevated toxic effect on all cell lines in an exponential manner. The latter observation suggests that after 5FU uptake into the cells, the drug is more likely to be anabolized through the uridylate metabolism rather than via the deoxythymidylate synthetic pathway. In the uridylate metabolic pathway, 5FU is used to generate precursors for FU-RNA synthesis. Thus, 5FU may have much more drastic damaging effects on RNA levels than it directly influences the TYMS catalyzed rate-limiting step of deoxythymidylate metabolism pathway.

TABLE 3 Relationship between tolerance to fluoropyrimidines and relative gene expressions detected in stable shRNA cell lines

shRNA cell lines	Drug treatment		Relative expression of genes		
	FUdR IC_{50} (μM)	5FU IC_{50} (μM)	DUT	TYMK	TK
Control	200.5 ± 3.5	21.1 ± 5.2	1	1	1
sc-shRNA	254.4 ± 1.2	21.5 ± 10.3	1.04 ± 0.08	1.0 ± 0.1	1.1 ± 0.01
dut-shRNA4X	5.0 ± 3.5	3.6 ± 1.3	0.06 ± 0.01	3.2 ± 0.3	1.9 ± 0.18
tymk-shRNA	170.1 ± 2.1	14.8 ± 3.5	0.80 ± 0.03	0.23 ± 0.02	1.2 ± 0.07

First two columns represent the IC_{50} values for 5FU and FUdR of the different stable shRNA cell lines. Further columns list relative expression levels of DUT, TYMK, and TK genes, as determined by quantitative-PCR. Results clearly show that DUT silencing by RNAi induces much elevated sensitivity against 5FU and FUdR, while TYMK silencing on its own induces much less sensitization against the same compounds. Experiments were conducted as described in the Materials and Methods section.

Silencing of the TYMK Gene and Its Effect on Expressions of Genes Involved in Thymidylate Metabolism

Since cellular response to DUT silencing resulted in an upregulation of TYMK expression, we aimed to examine the gene expression patterns of genes involved in thymidylate metabolism while exerting repression of this enzyme by RNAi. To achieve our target, we generated the tymk-shRNA stable cell line in which the TYMK gene was permanently repressed (Table 1 and Scheme 2). Interestingly, in an early experiment, deletion of the TYMK homologue cdc8 gene in yeast was found to be lethal.[25] However, in another study, repression of the TYMK gene by RNAi did not induce direct lethal consequences in human colon cancer cell lines.[20]

Quantitative PCR results revealed that silencing of the TYMK gene by RNAi achieved a significant ($P < 0.02$) 4-fold reduction of TYMK expression in the tymk-shRNA cell lines compared to the value of control cell lines (Figure 4A). To determine whether this reduction had any effect on expression levels of DUT, TK, DCTD, and TYMS genes, quantitative PCR measurements were performed to measure their mRNA levels in tymk-shRNA cells. No significant ($P > 0.05$) changes could be observed in case of the genes examined in the TYMK repressed cell line (Figure 4B). These data indicate that the 4-fold reduction in TYMK expression does not perturb the gene expression patterns of genes involved in thymidylate metabolism.

Effect of TYMK Silencing on Response to Fluoropyrimidines

To examine whether TYMK silencing could cause any enhanced sensitivity to fluoropyrimidines, tymk-shRNA and control cell lines (sc-shRNA and untransfected control) were exposed to 5FU and FUdR drugs in different experimental manners. According to the results of concentration-dependent responses, the IC_{50} values of the tymk-shRNS cell line showed a very slight

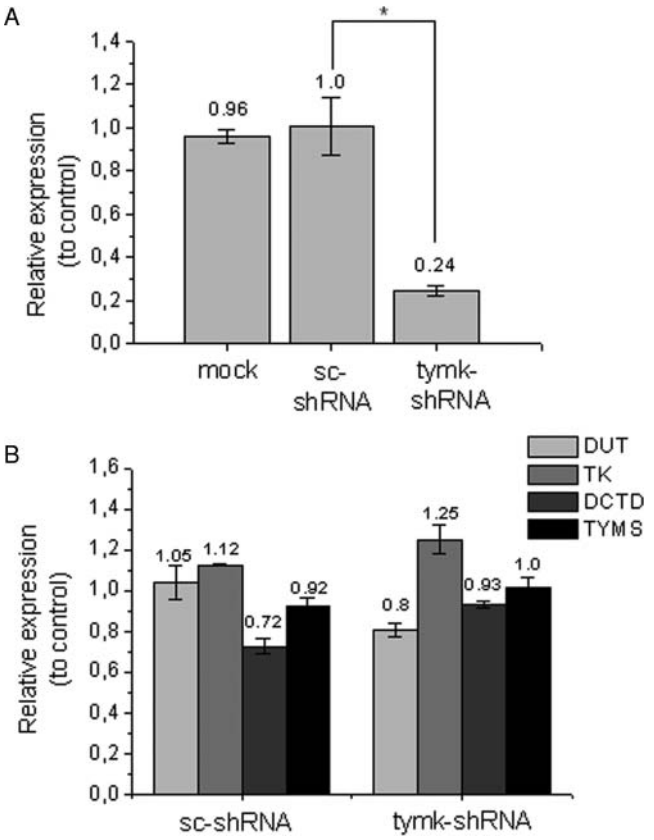


FIGURE 4 Downregulation of the TYMK gene by RNAi and its effect on expression patterns of genes involved in thymidylate biosynthesis in TR-HeLa cell. A) Downregulation of the TYMK gene detected by quantitative-PCR analysis. TYMK mRNA levels were quantified in tymk-shRNA cell line compared to control cell lines (mock, sc-shRNA) by using gene specific primer pair for amplification. B) Quantitative-PCR measurements show the effect of the TYMK gene silencing on expression patterns of genes involved in thymidylate biosynthesis in tymk-shRNA compared to control cell lines. DUT, TK, DCTD and TYMS genes mRNA levels were quantified by using gene specific primer pairs for amplification. For the quantitative-PCR experiments the cells were treated with tetracycline at the concentration of 1 μg/ml for 72 hours then harvested and prepared for analysis. $^*P < 0.02$. P value was calculated using a two tailed Student's t test. Data are presented as the mean ±SD. of two independent relative expression ratio determinations. Experiments were conducted as described in the Materials and Methods section.

sensitization to fluoropyrimidines compared to values of control cell lines (Figures 5A and 5B and Table 3). Furthermore, the viability of the repressed cell line was assessed in a time-dependent manner, at fixed concentrations of 5FU and FUdR drugs. Results showed a moderate but not significant increase in the sensitivity of tymk-shRNA cells for FUdR agents compared to control cells, while no noticeable sensitivity to 5FU could be detected in the case of the repressed cell line (Figures 5C and 5D). Data indicate that the 4-fold reduction of the TYMK gene expression does not robustly perturb the normal physiological function and does not induce any robust sensitization

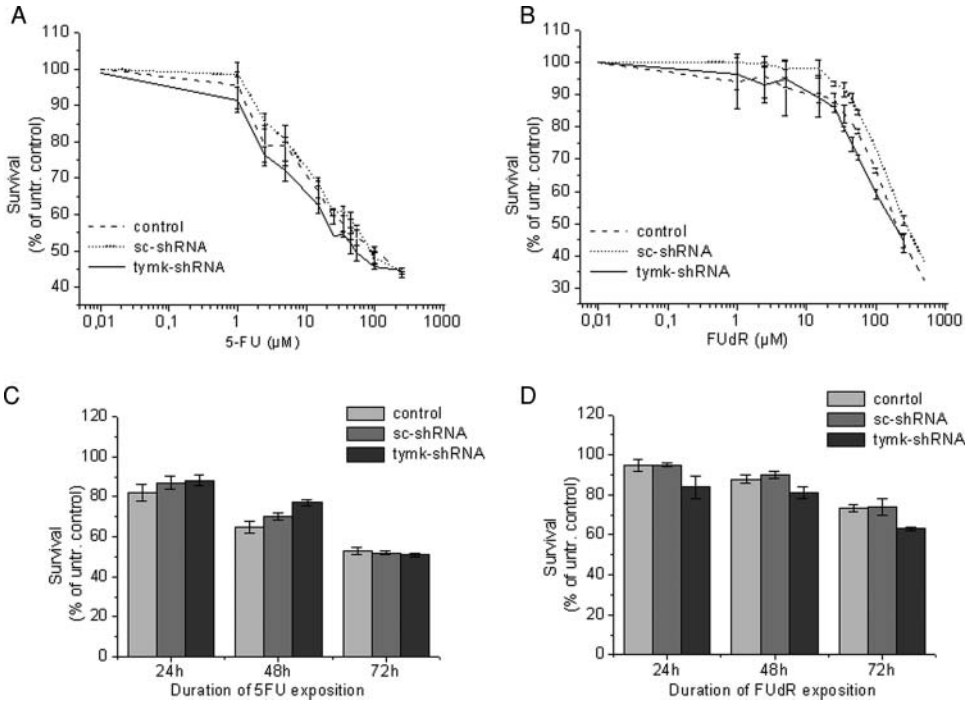


FIGURE 5 5FU and FUdR treatments of TR-HeLa cells with downregulation of the TYMK gene. A/B) Growth inhibition effects of 5FU (A) and FUdR (B) were estimated in a concentration-dependent manner on control (– – –), sc-shRNA (. . .), and tymk-shRNA (—) cell lines. Cells were administrated for 72 hours with various concentrations of 5FU (0–250 μM) or FUdR (0-500 μM). C/D) Growth inhibition effects of 5FU (C) and FUdR (D) were performed in time-dependent manner on control, sc-shRNA and tymk-shRNA cell lines as well. Cells were treated with discrete concentrations of 5FU (30 μM) or FUdR (30 μM) for different time intervals (0, 24, 48, 72 hours). Inhibition assay results are reported as percentage of untreated control for each cell lines. Measurements were conducted at the present of tetracycline at the concentration of 1 μg/ml. Data represent the mean ±SD of three independent determinations at each concentration. Experiments were performed as described in the Materials and Methods section.

of the cells to fluoropyrimidines. The observation suggests that in spite of significant TYMK silencing, the residual part of the enzyme could provide sufficient enzymatic activity for maintaining proper dTTP synthesis. In this respect, it is also to be noted that since the treatment with fluoropyrimidines results in inhibition of the TYMS enzyme, the substrate pool for TYMK will also be reduced in these cells.

dUTPase Activity Measurement by Colorimetric Assay

With respect to quantitative PCR and immunoblot results that indicated highly efficient silencing of the DUT gene in the dut-shRNA4X cell line, we also attempted to measure the dUTPase enzyme activity in whole cell extracts using a direct colorimetric assay. Results revealed that the actual dUTPase activity was relatively low in the dut-shRNA4X cells ($(0.17 \pm 0.04) \times$

10^{-3} unit/mg cell extract) (6.68%) as compared to the control cells ((2.63 $\pm$ 0.1) $\times$ 10^{-3} unit/mg cell extract) (100%). Data are in agreement with the mRNA and protein values of cell lines described above.

5F-Substitution Results in Subtle Changes in the Kinetic Mechanism of dUTP Hydrolysis by dUTPase

In the earlier work of Caradonna and Cheng,[26] the observation that human dUTPase can hydrolyze FdUTP was reported (cf. also Quesada-Soriano[27] for a recent report). We wished to characterize this catalytic property of human dUTPase in quantitative detail to provide further mechanistic insights. Adopting the method described in Toth et al.,[28] we measured relevant thermodynamic and kinetic parameters of FdUTP binding and hydrolysis (Figure 6 and Table 4). The stopped-flow traces recorded for the FdUTP hydrolysis reactions (Figure 6A) showed equivalent characteristics to that of unsubstituted dUTP hydrolysis, indicating that similar conformational changes occur during the enzymatic cycle (cf. Toth et al.[28]). The timescales of these conformational changes, however, differ between FdUTP and dUTP. FdUTP binding to and dissociation from the enzyme are 2-fold slower than that of dUTP, based on the first binding phase of the stopped-flow curves (Figure 6B). The conformational transition of the collision complex into the catalytically competent conformation, however, is twice as fast as in dUTP ($k_{ISO,\,obs} = 40 \pm 24\ s^{-1}$). We calculated this parameter from the second, concentration independent, exponential phase of the reaction curves shown in Figure 6A. Our single turnover experiments in Figure 6C (one substrate/enzyme can be converted) indicated that hydrolysis remains rate-limiting and occurs with similar rates to that of physiological dUTP. We also measured steady-state rates using the pH-indicator method[29] and observed similar enzymatic activities with dUTP and FdUTP (data not shown).

It is known that FdUMP acts as a potent inhibitor of thymidylate synthase.[30] Since dUMP is a product and a competitive inhibitor of dUTPase, we were curious to know whether the 5F-derivative displays an increased dUTPase affinity and more potent inhibition. To this end we measured the equilibrium binding of FdUMP to dUTPase (Figure 6D) and compared it to both the enzyme–dUMP and enzyme–FdUTP dissociation constants. We found that the 5F-substitution resulted in a slightly weaker dUMP binding, whereas the binding of FdUTP seems to be unaffected. We assessed the K_d for the enzyme–FdUTP complex by amplitude titration of the FdUTP binding transient kinetic experiments (Figure 6D inset). Moreover, calculation of the K_d from the kinetic parameters also results in a similar value ($K_d = k_{off}/k_{on} = 0.67\ \mu M$).

As a summary of our kinetic investigations, we report that the 5F-substitution on the uracil ring of the substrate does not change the kinetic

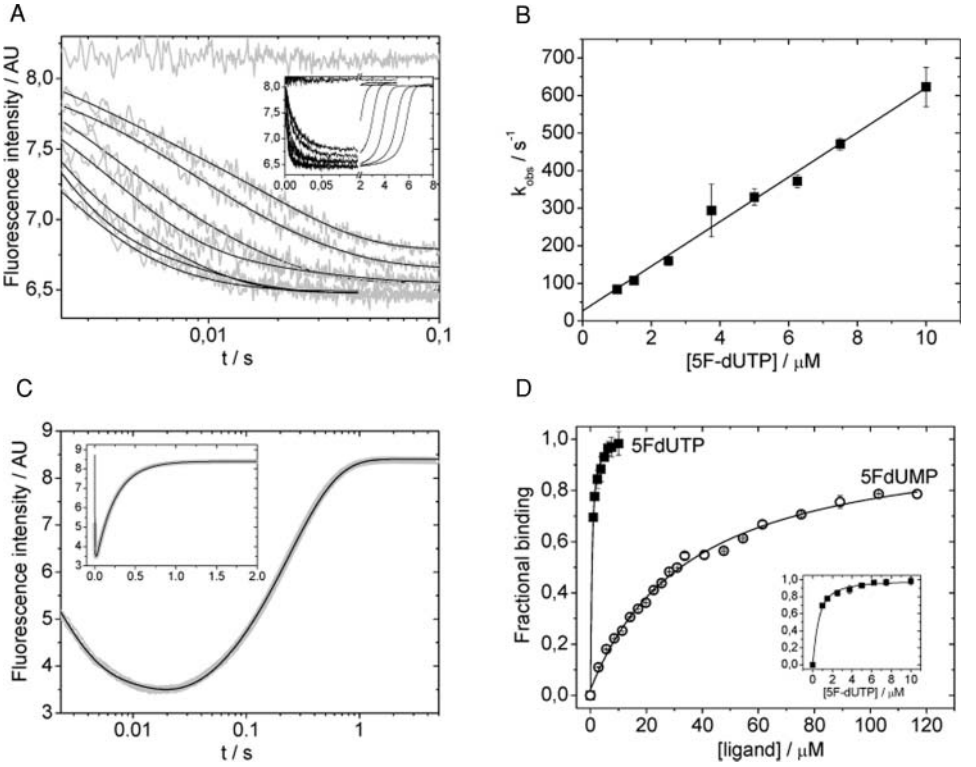


FIGURE 6 Kinetic characterization of the 5FdUTP hydrolysis cycle. A) Fluorescence time courses on the reaction of 0.5 μM hDUT^{F158W} upon mixing with buffer or with 1–10 μM 5FdUTP. The binding phases of the stopped-flow traces are shown in light gray, black lines are double exponential fits to the curves. Each curve represents the average of 5 traces and the entire series was repeated twice (Inset: complete time courses shown in linear time scale). B) 5FdUTP binding to hDUT^{F158W}. Rate constants (k_{obs}) extracted from the exponential fit to the initial phase of the reactions shown in panel A were plotted against 5F-dUTP concentrations. A linear fit to the data resulted in the following association and dissociation rate constants: $k_{on} = 59 \pm 3\ \mu M^{-1}s^{-1}$, $k_{off} = 27 \pm 15\ s^{-1}$. C) Single turnover hydrolysis curve upon mixing 22.5 μM hDUT^{F158W} with 13 μM 5FdUTP. A triple exponential function (black line) is fitted to the data points (gray circles). The third phase characterized with an increasing fluorescence corresponds to the single turnover hydrolysis rate constant k_{STO} ($=k_{cat}$) $= 4.2 \pm 0.01\ s^{-1}$. The inset shows the same curve in linear timescale. D) 5FdUTP and 5FdUMP equilibrium binding to hDUT^{F158W}. Fractional binding data for 5FdUTP was calculated from the amplitudes of the exponential fits to the binding phases (panel A). 5FdUMP binding was measured separately in an equilibrium fluorescence titration setup using 2.7 μM hDUT^{F158W} and increasing amounts of ligand. Quadratic fits to the titration curves yielded K_d (5FdUTP) $= 0.3 \pm 0.04\ \mu$M and K_d (5FdUMP) $= 46 \pm 4\ \mu$M. The inset displays the 5FdUTP binding curve again in a smaller concentration scale.

mechanism of dUTP hydrolysis by dUTPase. It alters the rate constants of the binding steps but not the overall affinity for the substrate. Product inhibition by FdUMP may be slightly weaker than that of dUMP. This important feature supports the suggestion that dUTPase interferes with the action of 5FU-based drugs in a dual manner at the FdUTP level: on one hand, it

TABLE 4 Comparison of kinetic parameters for FdUTP and dUTP hydrolysis as catalyzed by human dUTPase

	FdUTP substrate	dUTP substrate[28]
k_{cat} (s^{-1})	4.2 ± 0.01	6.8 ± 2
k_{on} ($\mu M^{-1}s^{-1}$)	59 ± 3	121
k_{off} (s^{-1})	27 ± 15	100
$k_{ISO, obs}$ (s^{-1})	40 ± 24	24 ± 6

k_{cat} is the catalytic rate constant, k_{on} and k_{off} are the rate constants for binding and dissociation of substrate to dUTPase, k_{ISO} is the rate constant for the conformational transition of the collision complex into the catalytically competent conformation. Data for dUTP are taken from the literature.[28]

produces the physiologically relevant thymidylate synthase inhibitor FdUMP, while on the other hand, it eliminates FdUTP, thereby preserving DNA from its incorporation.

CONCLUSION

dUTPase and TYMK are crucial for normal homeostatic function of the cell through dTTP synthesis, but both of these enzymes are also involved in metabolism of fluoropyrimidines (cf. Figure 6 and Lavie and Conrad[31] for TYMK). We have shown that the catalytic action of dUTPase on FdUTP is very similar to its action on the physiological substrate dUTP in most quantitative details (cf. Figure 6). The sanitizing action of dUTPase, that is, its crucial role in eliminating the nonphysiological nucleotide building block dUTP, is therefore similarly manifested for FdUTP also. Thus, depriving cells from the dUTPase-dependent preventive repair mechanism presumably leads to increases in both dUMP and FdUMP substitutions in the DNA. Repressing the dUTPase function has a much more pronounced sensitization effect toward FUdR, acting at the DNA level, as compared to 5FU, acting at both the RNA and DNA levels, indicating the dedicated role of dUTPase in DNA protection. dUTPase-silencing induced sensitization is, however, strong even against 5FU. This is important especially from the point of view of the clinically administered drug regimes. In addition to the sensitization effects, dUTPase silencing also induces significantly increased expression levels of TYMK and TK genes, which are both involved in the salvage pathway, but interestingly, not that of the TYMS gene. This result suggests that in addition to the relation previously proposed between dUTPase and TYMS in defining the mechanism of action of fluoropyrimidines.[15] the communication network needs to be broadened to include TK and TYMK as well. Our results present convincing evidence to the notion that actual cellular response to fluoropyrimidines will be determined by multiple factors in thymidylate metabolism.

MATERIALS AND METHODS

Materials

General chemicals were obtained from Sigma-Aldrich (Darmstadt, Germany). FdUTP and FdUMP were purchased from Jena Bioscience GmbH (Jena, Germany), antibiotics from Invitrogen (Carlsbad, CA, USA), and oligonucleotides from Sigma and Eurofins MWG Synthesis GmbH (Ebersberg, Germany).

Cell Culture

Human cervical adenocarcinoma, T-REx-HeLa (TR-HeLa) cell line (Invitrogen) was maintained and cultured according to the manufacturer's instructions. This cell line constitutively and stably expresses the Tet repressor (TetR) from the pcDNA6/TR TetR expression plasmid to facilitate rapid regulation of the vector-based inducible RNA interference system in a tetracycline-dependent manner. In our experimental conditions, exponentially growing cells were treated with tetracycline at the concentration of 1 μg/ml for 72 hours in order to induce the shRNA expression.

Establishment of RNAi Vector Constructs

In our RNAi experiments two different types of tetracycline inducible RNAi systems were applied: i) pSuperior vector (pSup.p) (Oligoengine, Seattle, WA, USA) for DUT gene silencing and ii) H1 RNAi Entry Vector (pEntr) (Invitrogen) for TYMK gene silencing. Four target sites for the DUT gene (nucleotides 200–219; 230–249; 350–369, 413–432; respectively) and one target site for the TYMK gene (nucleotides 509 to 527) were chosen within open reading frames of the genes (Table 1 and Scheme 1). Selection of target sites was partially based on previous studies.[5,17,19] Scrambled sequence was derived from the sequences of target sites of DUT and TYMK genes. We generated four DNA constructs directed against the DUT gene, pSup.p-200, −230, −350, −413, one against the TYMK gene, pEntr-509, and one for scrambled control, pEntr-sc (Table 1). Vector constructs were established according to the manufacturer's instructions.

Transfection Procedures for Generation of Stable Cell Lines

To generate stably transfected cell lines, we used Lipofectamin 2000 reagent (Invitrogen). The transfection procedures were carried out according to the manufacturer's instructions. For DUT gene silencing, pSup.p-200, -230, -350, and -413 DNA constructs were mixed in a 1:1:1:1 ratio before use. For TYMK silencing, the pEntr-509 vector; for mock generation, the pSup.p vector; and for generation of scrambled control, the pEntr-sc vector was

used on its own, respectively. Finally, four stable cell lines (dut-shRNA4X, tymk-shRNA, mock and sc-shRNA) were selected by respective antibiotics (Scheme 2).

RNA Isolation and Reverse Transcription

RNA was isolated using RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). RNA samples were quantified and analyzed by ND-1000 UV-vis spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA). An amount of 1 μg DNA free total RNA from each sample was reverse-transcribed using M-MuLV reverse transcriptase according the manufacturer's protocol (Fermentas, Ontario, Canada).

Quantitative PCR

Primer pairs for quantitative PCR (Q-PCR) were designed by DNA STAR/Primer Select software (Table 2). Real-time PCR reactions were performed in a Mx3005P QPCR System (Stratagene, Agilent Technologies, Inc., La Jolla, CA, USA) using Eva Green dye (Bioline, Csertex Kft., Budapest, Hungary) and ImmoMix (Bioline). The reaction conditions were optimized by determining the amplification efficiency for each primer pair and amplicon. To determine the amplification efficiency, serial dilutions of the cDNA template from control cells were used to produce a standard curve for each amplicon. Slopes were calculated by linear curve fitting. Efficiencies (E) were determined by applying the following equation $E = 10^{[-1/\text{slope}]}$. To standardize all the samples, GAPDH was used as an endogenous reference gene. We used a control (untransfected) cell line as a calibrator in our experiments. The target quantities were normalized to GAPDH and calibrated using values of controls defined as 1.0. Relative expression ratios of the examined genes were calculated using the comparative Ct method ($2^{-\Delta\Delta\mathbf{Ct}}$) with correction of PCR efficiency according to Pfaffl method.[32] Relative expression ratios of examined genes mean an n-fold difference relative to the calibrator. The quantitative analysis for each candidate gene was performed with two biological samples using independent cell cultures at different times. Each Q-PCR reaction for each gene was performed in technical triplicates (performed in three independent wells). After conducting the Q-PCR reactions, the means were calculated from the values measured for samples from independent wells. Standard deviations (SDs) were calculated from the means of Q-PCR measurements at different times using independent cell cultures. Significant alterations were calculated using the means of interest gene expression relative to means of gene expression detected in the scrambled-shRNA (sc-shRNA) cell line. A two-tailed student's t test was used for estimations of significance, with $P < 0.05$ considered to be significant.

Data were collected and quantitatively analyzed by the MxPro QPCR and OriginPro 7.5 softwares. Significant alterations were calculated for values of interest relative to values detected in the scrambled-shRNA cell line.

Generation of dUTPase Anti-Serum

Experiencing nonspecific characters of commercially available antibodies against human dUTPase, we generated a new anti-serum following the procedure described in Barabas et al.[33] Briefly, rabbits were immunized with recombinant human dUTPase.[34] Three immunising shots were given at time intervals of 2–3 weeks, first in complete then in incomplete Freund's adjuvant or in physiological saline.

Immunoblot Analysis of Proteins

Immunoblot analysis was done according to the method described previously.[35] First, the membranes were reacted with rabbit serum containing polyclonal anti-dUTPase antibodies (used at 1:5.000 dilution), mouse anti α-tubulin monoclonal antibody (Sigma-Aldrich), respectively. Secondly, the membranes were incubated with horseradish peroxidase conjugated secondary antibody: anti-rabbit IgG (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and anti-mouse IgG (Sigma), respectively.

Growth Inhibition Assay

Cell viability was evaluated by an AlamarBlue Cell Viability Assay Kit (Biotium, Inc., Hayward, CA, USA) according to the manufacturer's instructions. Exponentially growing cells in an equal number (2000 cells/well) were exposed for 72 hours to increasing concentrations of 5FU (0-250 μM) or FUdR (0-500 μM) in the presence of 1 μg/ml tetracycline. Alternatively, to examine the time-dependent character of drug treatments, growth inhibition assays were also carried out by applying discrete concentrations of 5FU (30 μM) and FUdR (30 μM) for different time intervals (0, 24, 48, 72 hours). The resulting fluorescence was measured using the micro-titer plate-reader WallacVictor-2 (Perkin Elmer, Waltham, Massachusetts, USA). IC_{50} values were defined as the applied drug (5FU, FUdR) concentration (μM) required for a half-maximal inhibitory effect on cell growth. IC_{50} values were calculated by nonlinear curve fitting using Origin Lab 7.5 software. Experiments were performed in triplicate and standard deviation values were calculated.

dUTPase Activity Measurement by Colorimetric Assay in Cellular Extracts

In order to examine the dUTPase activity of the dut-shRNA4X cells, we adopted the malachite green colorimetric assay as described previously.[36] Control and dut-shRNA4X cells (3×10^6) were treated with tetracycline at the

concentration of 1 μg/ml for 72 hours, then lyzed in buffer (50 mM Tris-HCl pH 7.4, 140 mM NaCl, 1 mM DTT, 1 mM protease inhibitor-cocktail) on ice. The protein extract concentration was determined by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories Ltd., UK). The procedure was conducted as described previously.[36] Briefly, the malachite green reagent was prepared freshly and contained malachite green, polyvinyl alcohol, ammonium molybdate, and distilled water (in a ratio of 2:1:1:2). The procedure was carried out onto 96 well-plate at room temperature. The reaction mixture (50 μl) contained 0.5 μg/μl protein extract, assay buffer (1 mM HEPES pH 7.5, 150 mM KCl, 1 mM $MgCl_2$) and 0.02 unit/μl pyrophosphatase enzyme. After starting the reaction with adding 40 μM dUTP, samples were taken and mixed with malachite green reagent in a ratio of 1:4. After five minutes incubation the optical density was determined using a ND-1000 UV-vis spectrophotometer. Quantitative values for dUTPase activity were derived from a standard curve. Each experiment was performed in duplicate and standard deviation values were calculated from two independent measurements at different times.

Recombinant Protein Expression and Reaction Conditions for dUTPase Enzyme Activity and Ligand Binding Studies

The His-tagged nuclear isoform of the human dUTPase Phe158Trp mutant construct ($hDUT^{W158}$) was expressed and purified as described previously.[28] This mutation allows direct characterization of dUTP hydrolysis kinetics and determination of dissociation constant of dUTPase-nucleotide complexes as described elsewhere.[28,34,37] The protein concentration was measured using the BioRad Protein Assay reagent and by UV absorbance ($\lambda_{280} = 15930\ M^{-1}cm^{-1}$ for $hDUT^{W158}$) and is given in monomers. All measurements involving $hDUT^{W158}$ were carried out in 20 mM HEPES pH 7.5 buffer, also containing 100 mM NaCl, 5 mM $MgCl_2$,[38,39] and 2 mM DTT (unless otherwise stated), at 20°C.

dUTPase Activity Measurement by Stopped-Flow Analysis

Measurements were done using a SX-20 (Applied Photophysics, UK) stopped-flow apparatus, as described before.[28] Tryptophan fluorescence was excited at 295 nm and emission was selected with a 320 nm long-pass filter. Time courses were analyzed using the curve fitting software provided with the stopped-flow apparatus or by Origin 7.5.

Determination of Dissociation Constant of the Complex Formed Between dUTPase and FdUMP

Fluorescence intensity titrations were recorded on a Jobin Yvon Spex Fluoromax-3 spectrofluorometer with excitation at 295 nm (slit 1 nm), emission at 353 nm (slit 5 nm) at 2.7 μM protein concentration. Fluorescence intensity data were plotted against the FdUMP concentration and fitted with

a quadratic function (Equation (1)) to extract the dissociation constant.

$$y = s + A * \left((c + x + K) - \sqrt{(c + x + K)^2 - 4 * c * x} \right) / 2 * c \qquad (1)$$

where $s = y$ at $c = 0$, $A =$ amplitude, $c =$ concentration of the constant component, and $K =$ dissociation constant.

Statistical Analysis

Data were analyzed using OriginPro7.5 software (www.originlab.com). Each experiment was repeated at least two or three times. Data represent the mean ±SD (standard deviation) of two or three determinations. A two-tailed student's t test was used for estimations of significance, with $P < 0.05$ considered to be significant.

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