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Modulation of human dUTPase using small interfering RNA

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Abstract

Deoxyuridine triphosphate nucleotidohydrolase (dUTPase) is responsible for maintaining low intracellular levels of dUTP, thus preventing the incorporation of dUTP into DNA. A 21 bp double-stranded RNA molecule (siRNAdUT3) targeted against motif 3 of human dUTPase resulted in a time- and dose-dependent decrease in dUTPase activity in transfected cells. dUTPase activity was reduced approximately 95 ± 5% in all cell lines tested 48 h after transfection with 2 μg siRNAdUT3 and it was maintained at this decreased level for at least 72 h. Down-regulation of dUTPase resulted in a significant increase in intracellular dUTP and a decreased proliferation of the transfected cells. Therefore, we conclude that dUTPase activity/expression can be down-regulated using siRNA specifically targeted to dUTPase mRNA and that this approach can be used to elucidate the role of dUTPase in DNA metabolism, as well as, to determine whether dUTPase is a valid target for drug development.

Keywords: dUTPase; DNA metabolism; dUTP; siRNA; Cell proliferation; Senescence; Cell cycle

Deoxyuridine triphosphate nucleotidohydrolase (dUTPase, EC 3.6.1.23) catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate. Not only does this reaction provide the precursor for de novo dTMP synthesis, but the decrease in the intracellular dUTP level reduces the probability that dUTP will be incorporated into DNA by DNA polymerases during replication and repair processes. Human dUTPase is a member of the homotrimeric family of dUTPases that include dUTPases from most eukaryotes, prokaryotes, and RNA viruses (reviewed in [1]). While considerable information has been obtained concerning the biochemical and structural properties of the homotrimeric dUTPases [1], information is limited concerning the regulation of dUTPase expression, as well as, whether the human dUTPase is essential for replication like the *Escherichia coli* [2] and *Saccharomyces cerevisiae* [3] dUTPases. Furthermore, several lines of evidence demonstrate that dUTPase may have a pivotal role in regulating the chemotherapeutic efficiency of certain thymidylate synthase (TS) inhibitors [4–7], suggesting that it could be a potential target for a novel class of inhibitors. The present study was designed to explore whether or not the human dUTPase could be targeted using an RNA interference approach and to determine whether down-regulation of the expression of dUTPase resulted in an increase in the dUTP pool.

Materials and methods

Cellular transfection systems. HeLa, HT29, and SW620 were grown and maintained in McCoy's 5A medium supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere. Cells were plated at a density of 4.0×10^5 cells/well (6-well plates) 24 h prior to transfection. RNA (21 bp, siRNAdUT3) that targeted a portion of domain 3 of human dUTPase (5′-GATTATAGGAAATGTTG-$3'_{339–357}$) (GenBank Accession No.: M89913) was synthesized with dTT overhangs by Qiagen-Xeragon (Germantown, MD). Cells were transfected with siRNAdUT3

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using the protocol described for the TransMessenger Transfection reagent (Qiagen) but using Metafectene (Biontex Laboratories) as the transfection reagent. A RNA (1–4 μg) to Enhancer R (μl) ratio of 1:2 was held constant. Cells were incubated at 37 °C for 4 h, washed with PBS, and then 2 ml of McCoy's 5A medium containing serum and antibiotics was added. At various times following transfection, cells were collected and used to determine dUTPase and UNG activities, dUTP/dTTP pools, as well as, dUTPase mRNA expression levels.

Enzyme assays. For determination of dUTPase and UNG activities cells were resuspended in a general extraction buffer (10 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM BME, and 20% v/v glycerol), lysed, and cell-free supernatants were prepared as described previously [8]. The supernatants were used immediately for the determination of dUTPase and UNG activities using assays described previously [8,9]. Protein was estimated using the Coomassie blue dye binding assay as described by Bio-Rad Laboratories using bovine serum albumin (BSA) as the standard.

Determination of dUTP and dTTP pools. dUTP and dTTP pools were determined as described by Horowitz et al. [10].

Northern analyses. Total cellular RNA was isolated from cells by the acid guanidinium isothiocyanate–phenol–chloroform extraction [11]. RNA (15 μg/lane) was size fractionated in 1% formaldehyde–agarose gels and the RNA was transferred by capillary blotting onto Hybond-N^+ membranes (Amersham, Arlington Heights, IL). Membranes were hybridized overnight at 42 °C in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.50% SDS, 10% dextran sulfate, 100 μg/ml herring-sperm DNA, and 10^6 dpm/ml of radiolabeled probe. The membranes were washed twice at 42 °C in 2× SSPE, 0.50% SDS followed by a high stringency wash at 65 °C in 0.50× SSPE, 0.50% SDS. The β-actin probe was isolated from a macrophage cDNA library by screening with a mouse β-actin oligonucleotide probe (Oncogene Science, Manhasset, NY). The human dUTPase probe was a 500 bp *Bam*HI/*Eco*RI DNA fragment obtained from plasmid pGEX2THdUT, which was kindly provided by Dr. E.M. McIntosh, University of Queensland. The gel-purified dUTPase DNA fragment as well as the β-actin DNA were radiolabeled with [^{32}P]dCTP using the High Prime Labeling Kit as described by the manufacturer (Roche, Mannheim, Germany). Membranes were exposed for 90 min at −80 °C to Kodak BioMax MR film. Autoradiographs were scanned using an Epson scanner and quantified using SigmaScan Pro 4 (SPSS, Chicago, IL). To account for differences in loading, dUTPase-specific signals were normalized to the β-actin signal.

Cell proliferation assays. The CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega, Madison, WI) was used to determine the effect of siRNAdUT3 treatment on cellular proliferation. Briefly, SW620, HT29, and HeLa cells were plated at a density of 10^4 cells/well (96-well plates) in complete McCoy's 5A medium. Cells were allowed to recover for 24 h before transfection with siRNAdUT3 (0.25 μg). Cells were incubated at 37 °C for an additional 48 h. The medium was then aspirated and 100 μl of fresh growth media was added. A mixture of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the electron coupling reagent phenazine methosulfate (PMS) was added to the culture medium and incubated for 90 min at 37 °C in a humidified CO_2 atmosphere. The quantity of formazan product, which is directly proportional to the number of viable cells in culture, was determined by the amount of absorbance measured at 490 nm. The absorbance at 490 nm was measured using an ELISA plate reader (BioTek FL600 Microplate Fluorescence Reader.

Statistical analyses. Statistical analyses were performed using Student's *t* test.

Results

To evaluate whether dUTPase activity could be decreased using siRNA, a 21 bp double-stranded RNA molecule targeted to a portion of domain 3 (nucleotides 339–357 of the nuclear isoform of human dUTPase) was synthesized and used to transfect HeLa, HT29, and SW620 cells. Initial studies demonstrated that the transfection of cells with siRNAdUT3 resulted in a dose-dependent decrease in dUTPase activity (Table 1). The greatest decrease in dUTPase activity occurred when cells were transfected with at least 2 μg siRNAdUT3 and this resulted in a statistically significant decrease ($p \geqslant 0.001$) in dUTPase activity in all three cell lines examined. There was no effect on UNG activity in any of the treated cells when compared to UNG activity in non-treated or transfection reagent treated cells (0.81 ± 0.13, 1.18 ± 0.24, and 1.46 ± 0.27 for HeLa, HT29, and SW620 cells, respectively) (data not shown). The decrease in dUTPase activity by siRNAdUT3 was also dependent upon time after transfection, with the maximum decrease occurring in all cell lines 48–72 h after transfection (data not shown).

To determine whether the decrease in dUTPase activity was due to a decrease in the amount of dUTPase mRNA, total RNA was purified from non-treated, Metafectene treated, and siRNAdUT3 transfected cells and analyzed by Northern hybridization using dUTPase and β-actin probes (Fig. 1). Comparison of the relative intensities of the dUTPase signal to the β-actin signal demonstrated that there was a decrease in dUTPase-specific mRNA when compared to β-actin. The decrease was approximately 22% and 31% in SW620 cells, and 82% and 91% in HeLa cells transfected with 2 and 3 μg siRNAdUT3, respectively.

A role of dUTPase in normal cell metabolism is to decrease the intracellular levels of dUTP. SiRNAdUT3 treatment had no effect on dTTP levels in either HeLa or SW620 cells, but there was a significant increase ($p > 0.001$) in dUTP levels in both cell lines (Fig. 2). Forty-eight hours after treatment there was a 12.75 ± 2.3 and 7.8 ± 1.7-fold increase in dUTP levels in HeLa

Table 1
The effect of siRNAdUT3 on dUTPase activity in HeLa, HT29, and SW620 cells

Treatment	dUTPase activity[a] Cell line		
	HeLa	HT29	SW620
None	16.88 ± 2.13	6.67 ± 1.18	22.04 ± 5.62
Metafectene	17.34 ± 3.52	6.22 ± 2.11	23.44 ± 4.26
1 μg	7.64 ± 2.34	4.36 ± 1.19	8.72 ± 1.31
2 μg	1.04 ± 1.66	0.50 ± 0.19	1.10 ± 0.88
3 μg	0.98 ± 1.28	0.62 ± 0.11	0.83 ± 0.92
4 μg	1.13 ± 0.55	0.44 ± 0.21	0.97 ± 0.15

[a] Cells were plated at a density of 4×10^5 cells/well 24 h prior to transfection. Cells were transfected as described in Materials and methods. Cells were collected 48 h after transfection and assayed for residual dUTPase activity. Values represent the average ± the standard deviation of a minimum of three experiments.

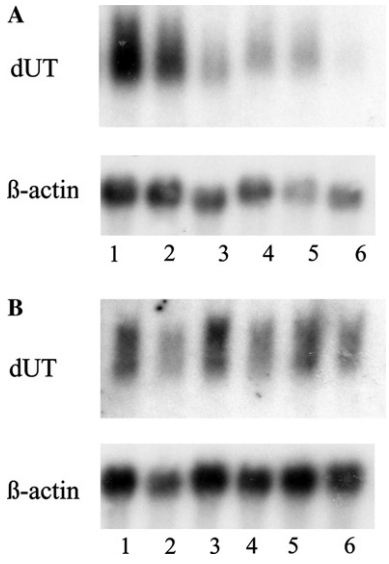

Fig. 1. Effect of siRNAdUT3 on dUTPase mRNA levels. HeLa and SW620 cells were plated at an initial density of 4×10^5 cells and 24 h after plating were transfected with 2 or 3 μg siRNAdUT3. Forty-eight hours post-transfection cells were collected, and RNA was extracted and analyzed as described in Materials and methods. (A) HeLa cells. (B) SW620 cells. Lane 1, non-treated control. Lane 2, Metafectene transfection reagent treated control. Lanes 3 and 4, 2 μg siRNAdUT3. Lanes 5 and 6, 3 μg siRNAdUT3.

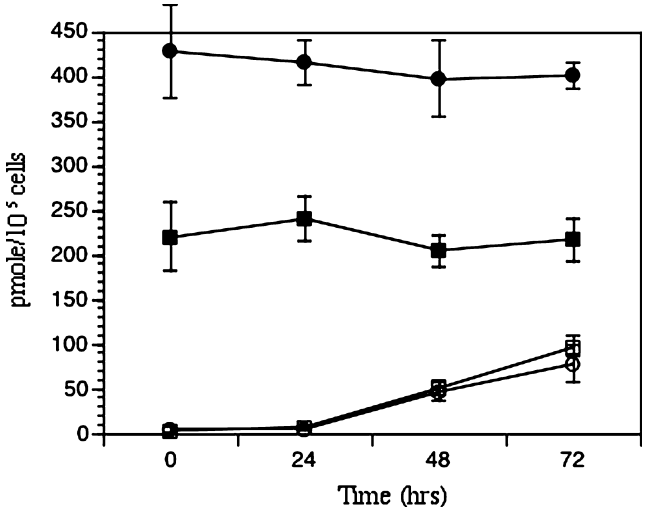

Fig. 2. Effect of siRNAdUT3 treatment on intracellular dUTP and dTTP levels. Cells were plated at an initial density of 4×10^5 cells and 24 h after plating were transfected with 2 μg siRNAdUT3. At 24 h intervals cells were collected and used to determine dUTP and dTTP concentrations as described in Materials and methods. Values represent the average ± the standard deviation of at least three independent experiments. HeLa dTTP (■), HeLa dUTP (□), SW620 dTTP (●), and SW620 dUTP (○).

and SW620 cells, respectively, when compared to controls and this elevation in dUTP increased to 24.5 ± 2.0 and 13.0 ± 7.4-fold by 72 h following treatment.

To assess the fate of cells transfected with siRNAdUT3, HeLa, HT29, and SW620 cells were transfected

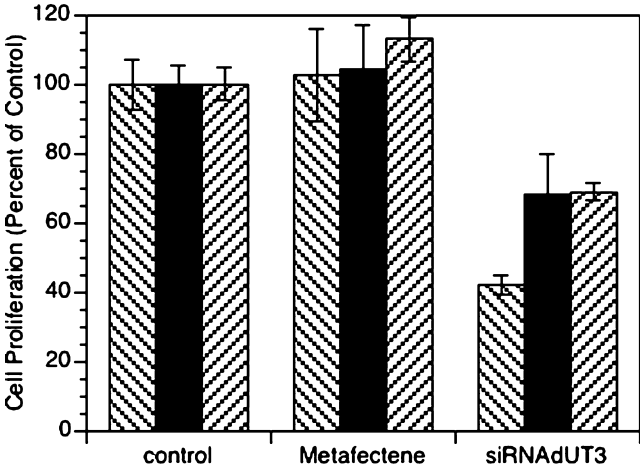

Fig. 3. Effect of siRNAdUT3 on cellular proliferation. Cells were plated and treated with either Metafectene or Metafectene with siRNAdUT (2 μg) as described in Materials and methods. Forty-eight hours after treatment cellular proliferation was determined using the MTS assay. Values for cellular proliferation were extrapolated from a standard curve established from the absorbencies of known cell concentrations. Values were standardized to the non-treated controls (100%). Values represent the average ± the standard deviation of four independent experiments. HeLa (▨), SW620 (■), and HT29 (▨).

with 2 μg siRNAdUT3 and assayed for cellular proliferation 48 h after transfection using the MTS assay. Cell proliferation was decreased in all transfected populations when compared to non-treated and transfection reagent treated cell populations (Fig. 3). The largest decrease in cell proliferation ($57.9 \pm 2.6\%$) was observed in HeLa cells. Conversely, cell proliferation was decreased $31.1 \pm 2.5\%$ and $32.1 \pm 12.21\%$ in HT29 and SW620 cells, respectively.

Discussion

The results of this study demonstrate that siRNA targeted to a portion of motif 3, which is known to be part of the catalytic site(s), of the human dUTPase resulted in a time- and dose-dependent decrease in dUTPase activity in treated cells when compared to non-treated or transfection reagent treated controls. The decrease in dUTPase activity correlated with an increase in intracellular dUTP levels. The decrease in dUTPase activity correlated with a down-regulation of dUTPase-specific mRNA at least in the case of HeLa cells. While there was down-regulation of dUTPase-specific mRNA in SW620 cells treated with siRNAdUT3, the total down-regulation did not correlate with decreased dUTPase activity. This may reflect a more rapid turnover of dUTPase in SW620, increased stability of dUTPase-specific mRNA in SW620 cells, or inefficient translation of mRNA in these cells following siRNA treatment. Studies are presently being performed to address these possibilities.

The decrease in cell proliferation following siRNAdUT3 treatment was somewhat surprising. Human cells contain mitochondrial (dUT-M) and nuclear (dUT-N) isoforms of dUTPase that are encoded by the same gene through the use of alternative 5′exons [12]. Since the isoforms are identical except for a short region located on the N-termini, siRNAdUT3 should result in the post-transcriptional degradation of the mRNA encoding dUT-N and dUT-M. It is possible that the decrease in proliferation is the result of inhibition of dUT-M, which may have a toxic effect on cellular proliferation. However, we do not believe that this is likely since the nuclear isoform is the most predominant dUTPase expressed in cells, and since the siRNAdUT3 treated cells retained viability, suggesting that the cells had arrested and were senescent.

The amount of dUTP incorporated into DNA by DNA polymerases is dependent upon: (i) the ability of the polymerase to discriminate between dUTP and dTTP, (ii) the intracellular levels of dUTP when compared to dTTP (dUTP:dTTP pool ratio) and (iii) dUTPase activity. There is essentially no discrimination between dUTP and dTTP by DNA polymerases [13] and in actively replicating human cells where the dUTP pool is normally low when compared to the dTTP pool, there are approximately 2000 dUTP molecules incorporated into human DNA per day [14]. Our results demonstrate that the siRNAdUT3 decreased dUTPase activity in treated cells, leading to an increase in intracellular dUTP levels, which causes a dUTP:dTTP pool imbalance and results in the increased incorporation of dUTP into DNA. This suggests that the decreased cellular proliferation is due to the increased incorporation of dUTP in DNA accompanied by the excision of uracil from DNA. Similar results have been obtained following the treatment of *S. cerevisiae* mutants that produced suboptimal amounts of dUTPase with antifolates [7].

There are accumulating data which suggest that dUTPase could be used as a target for the development of a specific class of chemotherapeutic agents that could be used either alone or in combination with existing agents to treat a variety of diseases. However, only few inhibitors have been described that inhibit dUTPase in vitro, and most of these are targeted against the monomeric dUTPases encoded by the herpesviruses [15,16]. Structural data have been obtained for several of the homotrimeric dUTPases, including the human enzyme [17], which could lead to the development of compounds that specifically inhibit dUTPases. However, we do not believe, at this time, that it is possible to rationally develop nucleotide analogs that effectively inhibit the activities of dUTPase in vivo. This is because these analogs have to be delivered to cells as nucleosides and be metabolically activated. The intermediates formed during metabolic activation may function as substrates and/or inhibitors for several enzymes involved in thymidylate and DNA biosynthesis. The complexities of such reactions make accurate analysis and interpretation of the data difficult if not impossible. Therefore, alternative approaches are required to inhibit dUTPase. While additional studies need to be performed to determine how siRNAdUT3 decreases cell proliferation, the results of this study demonstrate that dUTPase activity can be decreased greater than 95% in a variety of cell types and demonstrate that such an approach can be used to determine whether dUTPase can be successively used as a target and to determine its roles in normal cellular metabolism, as well as, in regulating the chemotherapeutic effectiveness of other agents.

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