Small Interfering Double-Stranded RNAs as Therapeutic Molecules to Restore Chemosensitivity to Thymidylate Synthase Inhibitor Compounds

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ABSTRACT

RNA interference is a post-transcriptional mechanism by which double-stranded RNA specifically silence expression of a corresponding gene. Small interfering double-stranded RNA (siRNA) of 21–23 nucleotides can induce the process of RNA interference, or RNAi. Studies from our laboratory have shown that translation of thymidylate synthase (TS) mRNA is controlled by its own protein end-product TS in a negative autoregulatory manner. Disruption of this process gives rise to increased synthesis of TS and leads to the development of cellular drug resistance to TS-targeted compounds. As a strategy to inhibit TS expression at the mRNA level, siRNAs were designed to target nucleotides 1058–1077 on human TS mRNA. Transfection of TS1058 siRNA into human colon cancer RKO cells resulted in a dose-dependent inhibition of TS expression with an IC50 value of 10 μM but had no effect on the expression of α-tubulin or topoisomerase I. Inhibition of TS expression by TS1058 was maximal at 48 h and remained suppressed for up to 5 days. Pretreatment of RKO cells with TS1058 siRNA suppressed TS protein induction following exposure to raltitrexed. Continued, TS1058 restored chemosensitivity of the resistant RKO-HTStet cell line to various TS inhibitor compounds. On treatment with TS1058, IC50 values for raltitrexed, 1843U89, and 5-fluoro-2'-deoxyuridine decreased by ~15–16-fold. These studies suggest that TS-targeted siRNAs are effective inhibitors of TS expression and may have therapeutic potential by themselves or as chemosensitizers in combination with TS inhibitor compounds.

INTRODUCTION

RNA interference (RNAi) is a novel regulatory process in which double-stranded RNA (dsRNA) induces the specific degradation of its target mRNA (1–3). This evolutionarily conserved process maintains genomic integrity of cellular organisms by silencing transposons and guarding against exogenous viral infection. Recent studies have shown involvement of the RNAi machinery with cellular processes such as chromosomal separation, histone methylation, and association with the fragile X protein (4–6). Fire et al. (7), who showed that dsRNA injected into Caenorhabditis elegans resulted in specific interference of cellular gene expression, first coined the term “RNAi.” The inhibitory effect was much greater than that observed with injection of either individual RNA strand. RNAi and closely related mechanisms, such as cosuppression and quelling, have been observed in fungi, plants, Drosophila, and mammals (1). The molecular mechanism involves cleavage of the dsRNA by an Rnase III-like endonuclease to small 21–25 nucleotide (nt) dsRNAs (8–10). These small interfering dsRNAs (siRNAs) are bound in an endonuclease RNA-induced silencing complex, and the antisense strand then is used as a guide to target complementary mRNAs for degradation (11). RNAi gained rapid acceptance as a tool for studying gene function in C. elegans and Drosophila. Application of RNAi in mammalian systems was limited initially because of nonspecific effects of long dsRNAs (12–14). However, since the discovery that siRNAs can be introduced into mammalian cells, thereby bypassing the nonspecific effects associated with dsRNAs, this mechanism has become widely used to investigate the regulation of gene expression (15). In addition, siRNAs may be developed as novel agents that target critical signaling pathways involved in human disease.

Thymidylate synthase (TS) is a folate-dependent enzyme that catalyzes the reductive methylation of dUMP by the reduced folate dTMP and dihydrofolate (16). Once synthesized, dTMP then is additionally metabolized intracellularly to the dTTP triphosphate form, an essential precursor for DNA synthesis. Although dTTP also can be formed through the salvage pathway, as catalyzed by thymidine kinase, the TS-catalyzed reaction provides for the sole intracellular de novo source of dTMP. Given its central role in DNA biosynthesis and that inhibition of this reaction results in cessation of cellular proliferation and growth, TS represents an important target for cancer chemotherapy (17–19).

In addition to its catalytic activity, TS functions as an RNA binding protein (20, 21). Studies from our laboratory have shown that TS protein binds with high affinity to two cis-acting sequences on its own mRNA. Binding of TS to either of these elements results in translational repression. This interaction between TS protein and its mRNA represents an efficient mechanism to control the cellular levels of TS. Disruption of this normal autoregulatory process results in an acute induction of TS and the rapid development of resistance in response to exposure to TS inhibitors such as 5-fluorouracil and the antifolate analogue raltitrexed. In support of this model, in vitro and in vivo studies have shown acute induction of TS protein with no corresponding change in TS mRNA levels on treatment with various TS inhibitors (22–24).

In the present study, we demonstrate that siRNAs effectively induce the process of RNAi in human colon cancer RKO cells. Treatment with natural 2'-OH siRNAs directed against nt 1058–1077 on human TS mRNA specifically and effectively inhibits the expression of TS mRNA and TS protein in RKO cells. In addition, siRNA treatment suppressed TS protein induction following exposure to the TS inhibitor compound raltitrexed. We demonstrate that treatment with siRNAs restored chemosensitivity to resistant, TS-overexpressing, RKO-HTStet cells against several clinically relevant TS anticancer agents. The potential therapeutic application of siRNAs as single agents and in combination with TS inhibitor compounds for the treatment of human cancers is discussed.

MATERIALS AND METHODS

siRNA Synthesis. siRNA duplexes were designed to target sequences on human TS mRNA corresponding to nt 1058–1077 (5'-GGAAUAUUGUCAGUUAGG-3'). The selected sequence was screened in a BLAST search against all of the known human genes to verify that only human TS mRNA was targeted. siRNA duplexes were obtained from Dharmacon (Lafayette, CO). Each RNA contained two additional 2'-deoxymethylidines nts on the 3’ end. In addition, a mismatch siRNA, TS1058M10, and two control siRNA duplexes, GL2 and SCR1, were obtained from Dharmacon.

Cell Culture. The human colon cancer cell lines RKO and RKO-HTStet were maintained in 75-cm² tissue culture flasks (BD Bioscience, San Jose, CA)
in growth medium containing RPMI (1640; Invitrogen, Carlsbad, CA) with 10% dialyzed fetal bovine serum (Invitrogen).

A stably transfected, tetracycline-inducible RKO subline, which overexpresses human TS protein, was established by transfection with plasmid pUHDI72–Ieo using lipofectin. This plasmid constitutively expresses the Tet activator protein. Cells were grown in RPMI 1640 medium containing 10% dialyzed fetal bovine serum and 600 μg/ml geneticin. After 3 weeks of growth, colonies were selected individually and expanded into cell lines. The full-length human TS cDNA (nt 1–1524) was isolated from the plasmid pCEHTS by PCR amplification and cloned into pTRE2hyg, a tetracycline-responsive plasmid (Clontech, Palo Alto, CA). The plasmid pTRE2hyg-HTS-THS was transfected into RKO cells expressing the Tet activator protein by Eufectin 7 (JBL Scientific, San Luis Obispo, CA). Colonies were selected in 600 μg/ml genetin and 300 μg/ml hygromycin. Each clone was treated with 1 μg/ml doxycycline for 24 h. Clones with elevated levels of induced TS protein expression, as determined by Western blot analysis, then were selected, and the clone that yielded the highest level of induced TS expression was termed RKO-HTStet.

siRNA Transfection. Human colon cancer RKO cells were plated in 25-cm² flasks in 3 ml RPMI 1640 at a density of 1.5 × 10⁵ cells per flask. siRNAs were complexed with the cationic lipid Oligofectamine (Invitrogen) in OPTI-MEM medium, as described by the manufacturer’s protocol. In brief, 4 μl of Oligofectamine were added to 21 μl OPTI-MEM medium and allowed to sit at room temperature for 5 min. siRNA duplexes were added to 275 μl OPTI-MEM medium. Diluted Oligofectamine was added to the diluted siRNA and incubated for 15 min at room temperature. Aliquots (300 μl) then were added to the T25 flasks. siRNA concentrations cited herein are in a final total volume of 3.3 ml. After 48 h, cells were trypsinized and washed twice with ice-cold PBS. Cell pellets were stored at −80°C for later use.

For cell proliferation assays, RKO-HTStet cells were plated in 12-well plates in 1 ml RPMI 1640 at a density of 1 × 10⁵ cells/well. Oligofectamine (1 μl) was added to 14 μl OPTI-MEM and allowed to sit for 5 min at room temperature. This solution then was added to the diluted siRNA (85 μl) and allowed to incubate for 15 min before addition into the wells.

Western Immunoblot Analysis. RKO cells were plated, transfected, and harvested as described previously. Cell pellets were resuspended in cell lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% Igepal (Sigma-Aldrich Co., Milwaukee, WI), 0.5% deoxycholic acid, and 0.1% SDS] containing freshly added phenylmethylsulfonyl fluoride and protease inhibitor mixture (Sigma, St. Louis, MO). Suspensions were incubated at 4°C for 20 min and centrifuged at 10 min at 4°C. Protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA). Equivalent amounts of protein (30 μg) from each cell lysate were resolved on SDS-PAGE (10–12.5% acrylamide) using the method of Laemmli (25). Gels were electrophoretically transferred onto nitrocellulose membranes (0.2 μm; Bio-Rad), and membranes then were incubated in blocking solution (1× PBS, 0.1% Tween-20, and 5% nonfat dry milk powder) for 2 h at room temperature. Membranes were incubated for 1 h with primary antibodies at the following dilutions: anti-TS105 monoclonal antibody, 1:2,000; anti-α-tubulin monoclonal antibody, 1:30,000 (Amersham, Piscataway, NJ); and antitopoisoenzyme I monoclonal antibody, 1:2,000 (a gift from Dr. Yung-chi Cheng, Yale University, New Haven, CT). After five 10-min washes in 1× PBS and 0.1% Tween-20, membranes were incubated with a dilution of 1:2,000 of horseradish peroxidase-conjugated secondary antibody (IgG goat antimouse; Bio-Rad) for 1 h at room temperature. After an additional five 10-min 1× PBS and 0.1% Tween-20 washes, membranes were processed by the enhanced chemiluminescence method (SuperSignal West Pico substrate; Pierce, Rockford, IL), and protein bands were visualized by autoradiography. Quantitation of signal intensities was performed using densitometry on a Hewlett-Packard ScanJet 5370C (Palo Alto, CA) with NIH image 1.62 software.

Cell Proliferation Assays. RKO-HTStet cells were plated in 12-well plates in the presence or absence of 1 μg/ml doxycycline. siRNA–Oligofectamine complexes then were added to the wells on the following day. After a 24-h incubation, TS inhibitor compounds, including raltitrexed (ZD1694), 1843U/89, and 5-fluoro-2’-deoxyuridine (FdUrU), were added to the wells at the indicated concentrations. After an additional 72 h, cells were trypsinized, and the cell number was determined by a Coulter counter (Beckman Coulter, Fullerton, CA) and by the trypan blue dye exclusion method.

RESULTS

We investigated the ability of siRNAs to inhibit the expression of TS in RKO cells. As seen in Fig. 1, TS1058 siRNA, which targets the 3’-UTR immediately downstream of the translational stop site (nt 1058–1077), inhibited the expression of TS protein in a dose-dependent manner with 50% inhibition being observed at 10 pm and maximal inhibition (>95%) at 300 pm (Fig. 1, Lane 4 and 7, respectively). The expression of two control proteins, α-tubulin and topoisomerase I, was unaffected by TS1058 siRNA treatment. Treatment with a control GL2 siRNA, at a concentration of 10 nm, had absolutely no effect on levels of TS or the control proteins (Fig. 1, Lane 10). Expression of TS protein remained unchanged by GL2 concentrations up to 100 nm (data not shown). We also investigated the effect of TS1058 siRNA on TS enzyme activity using the TS catalytic assay and the FdUMP radioenzymatic binding assay (26). The reduction in TS protein expression, as determined by Western blot analysis, corresponded exactly with inhibition of TS enzyme activity and FdUMP binding activity (Fig. 1).

Previous studies from our laboratory had shown that binding of TS protein to its own TS mRNA resulted in translational repression (20, 27). Disruption of this normal autoregulatory process gave rise to an acute induction of TS and the acute development of resistance in response to exposure to TS inhibitors such as 5-fluorouracil and raltitrexed (ZD1694; Refs. 23 and 26). In attempts to overcome this resistance mechanism, we investigated whether TS-targeted siRNA duplexes could prevent this induction of TS protein. As seen in Fig. 2A, RKO cells were treated for 24 h with ZD1694, TS1058 siRNA, or a combination of these two molecules. Treatment of RKO cells with 2 nm ZD1694 for 24 h resulted in a more than twofold induction of TS protein (Fig. 2B). This induction was prevented when RKO cells were pre-treated with 1 nm TS1058 siRNA for 24 h before treatment with ZD1694. However, when ZD1694 was administered first, followed by TS1058 siRNA, TS protein remained elevated at levels observed with ZD1694 treatment alone. Additionally, when RKO cells were treated with TS1058 siRNA and ZD1694 simultaneously during the second 24-h period, induction of TS protein still was observed, albeit at a slightly lower level than that observed with ZD1694 treatment alone. However, when RKO cells were incubated together with ZD1694 and TS1058 siRNA during the first 24-h period, TS protein levels decreased by nearly 70% compared with control, untreated levels. As an important control, treatment of RKO cells with the control GL2 siRNA (1 nm) for 24 h before the addition of ZD1694 was unable to abrogate the induction of TS protein.

![Fig. 1. Western blot analysis of RKO cells after treatment with a thymidylate synthase (TS)-targeted small interfering double-stranded RNA (siRNA).](image-url)

Fig. 1. Western blot analysis of RKO cells after treatment with a thymidylate synthase (TS)-targeted small interfering double-stranded RNA (siRNA). Cells were incubated in the absence (Lane 1) or presence (Lanes 2–10) of siRNA/Oligofectamine complexes for 48 h and then harvested and processed for Western blot analysis as described in “Materials and Methods.” Lanes 2–9 correspond to treatment with Oligofectamine complexes containing 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 10 nm TS1058 siRNA, respectively. Lane 10 corresponds to 10 nm GL2 siRNA. TS catalytic enzyme activity and FdUMP binding assays were performed as described previously (26). Assay values are based on untreated RKO cells, which represent 100%.
We next performed a time course experiment to determine the effect of siRNA treatment on levels of TS protein following the 48-h point. As seen in Fig. 2C, the expression of TS protein remained elevated when RKO cells were treated only with ZD1694. However, TS levels rapidly decreased during the next 12-h period when RKO cells were treated with either ZD1694 being administered first before the TS1058 siRNA or when ZD1694 and TS1058 siRNA were administered concomitantly. Taken together, these results suggest that TS-targeted siRNAs are able to prevent the induction of TS protein in response to exposure to TS inhibitor compounds.

The activity of TS1058 siRNA was investigated subsequently in a tetracycline-inducible, TS-overexpressing cell line, RKO-HTStet. This particular cell line had been established after stable transfection of the human TS cDNA under the control of a tetracycline-inducible promoter into parent RKO cells. Following treatment of RKO-HTStet cells with 1 µg/ml doxycycline for 24 h, levels of TS protein, as determined by Western immunoblot analysis, were 15-fold higher compared with parent RKO cells (Fig. 3A, Lane 3 versus Lane 2). Transfection of RKO-HTStet cells with 1 nM TS1058 siRNA resulted in a 50% reduction in TS protein expression (Fig. 3A, Lane 4). On treatment with 10 nM TS1058, levels of TS protein returned to the same baseline levels observed in noninduced cells (Fig. 3A, Lane 6 versus Lane 2). In contrast, the control GL2 siRNA had no effect on TS expression (Fig. 3A, Lane 7).

Fig. 2. Effect of TS1058 in combination with ZD1694 on induction of thymidylate synthase (TS) protein. A, schematic of experimental design: cells were incubated with either 1 nM TS1058 small interfering double-stranded RNA (siRNA; TS), 1 nM GL2 siRNA, or 2 nM ZD1694 (ZD) for a 24-h period. The number in parenthesis indicates whether cells were incubated during the first 24-h (1) or the second 24-h period (2). Culture medium was replaced after the first 24-h incubation. B, cells were harvested after 48 h and processed for Western blot analysis as described in “Materials and Methods.” C, RKO cells that were treated with ZD1694(2) (●), ZD1694(1) (○), TS1058(2) (●), or TS1058(2) + ZD1694(2) (□) were harvested at the following times after the 48-h incubation: 0, 12, 24, and 36 h. TS protein levels were determined by Western blot analysis. TS protein induction values represent the mean ± SE from densitometric analysis of Western blot analyses from four experiments. TS protein content in untreated RKO cells was set to 1. *P ≤ 0.005 versus ZD(2)

Fig. 3. Effect of TS1058 siRNA on thymidylate synthase (TS) protein expression and cellular proliferation in RKO-HTStet cells. A, cells were incubated in the absence (Lanes 1–5) or presence (Lanes 6–7) of small interfering double-stranded RNA (siRNA)/Oligolectamine complexes for 48 h and then harvested and processed for Western blot analysis. Lane 1 contains extracts from untreated parental RKO cells. Lane 2 contains extracts from RKO-HTStet cells without doxycycline. Lanes 3–7 contain extracts from RKO-HTStet cells treated with 1 µg/ml doxycycline. Lanes 4–7 are treated with Oligolectamine complexes containing 1, 3, and 10 nM TS1058 siRNA, respectively. Lane 7 corresponds to treatment with 10 nM GL2 siRNA. B, cells were treated with 1 µg/ml doxycycline. On the next day, cells were incubated with Oligolectamine in the absence or presence of siRNA (1 nM TS1058 or 1 nM GL2). After 24 h, various concentrations of TS inhibitor compounds [ZD1694; (D), 1843U89; (E), FdUrd] were added to the cells. After an additional 72 h, cell number was determined, and drug concentrations that inhibit 50% cellular proliferation (IC50) were estimated. C, in the presence of 1 µg/ml doxycycline, RKO-HTStet cells were treated with various concentrations of TS1058. After 24 h, various concentrations of ZD1694 were added to the cells. After an additional 72 h, cell number was determined, and IC50 values were calculated. Values represent the mean ± SD from three to five experiments.
DISCUSSION

In this report, we investigated the effect of siRNAs on expression of TS protein and TS mRNA in human colon cancer RKO cells. Our studies reveal that siRNAs significantly inhibit expression of TS protein for an extended period. This effect on TS appears to be specific because other important cellular proteins, including α-tubulin and topoisomerase I, were unaffected by TS-directed siRNA treatment. Additionally, TS1058 siRNA was able to inhibit RKO cellular proliferation cells by 30%. This limited effect of cell growth was surprising initially given the significant degree of down-regulation of TS protein (>95%). However, previous studies by Keyomarsi and Moran had suggested that >98% inhibition of TS enzyme activity was required for TS enzyme inhibition to translate into inhibition of cell growth and proliferation (28). Similar observations were reported with antisense DNA molecules targeting TS mRNA, whereby oligodeoxyribonucleotides effectively inhibited TS mRNA levels but had little to no effect on cell proliferation (29). Our laboratory had shown previously that modified antisense oligoribonucleotides (ORNs) inhibited the same RKO cells with IC50 values in the 200-nM range (30). However, control sense and mismatched ORNs, which had no effect on TS protein expression, resulted in similar IC50 values as the antisense ORN. This result suggested that the effect on inhibition of cell growth was not caused entirely by specific suppression of TS expression.

One of the major obstacles in cancer therapy is the development of cellular resistance to cancer chemotherapy. In vitro, in vivo, and clinical studies have shown that exposure to TS inhibitor compounds results in acute induction of TS expression (22–24, 31). Our laboratory and others have proposed that this induction process leads to the development of cellular drug resistance to TS inhibitors. Two well-established regulatory processes that mediate the acute induction of TS expression are increased translation of TS mRNA and enhanced stability of the TS protein (32). Our observation that the induction of TS protein was not abrogated when RKO cells were first treated with ZD1694 followed by the TS-targeted siRNA suggests that TS induction may be caused by stabilization of TS protein. Furthermore, levels of TS protein were induced after 24 h of treatment with the combination of ZD1694 and TS1058 siRNA. However, when cells were treated with this combination during the first 24 h, TS protein levels decreased by nearly 70% compared with control levels. This finding suggests that the inability of the TS-targeted siRNA to block the ZD1694-mediated induction of TS protein may be caused by a timing effect as to when cells are treated with the siRNA and TS inhibitor. Under these conditions, the final expression of TS appears to be the net result of two competing cellular events: the first is the acute induction of TS protein by ZD1694, whereas the second is the active degradation of TS mRNA by TS1058 siRNA. To investigate this issue further, we conducted a time course experiment to determine the effect on TS protein levels after the 48-h point. Our results demonstrate that regardless of whether ZD1694 was administered first followed by the siRNA or administered together with the siRNA, the expression of TS decreased rapidly after an additional 12 h. This result then would suggest that the TS protein is not stabilized under these specific conditions. Thus, our findings show that TS1058 siRNA can prevent induction of TS protein in RKO cells following incubation with raltrexed. Furthermore, they provide strong evidence that siRNAs can be used to abrogate effectively this acute resistance mechanism.

Our laboratory and others have shown that tumors overexpressing TS protein, either acutely or chronically, are resistant to TS inhibitor compounds (33, 34). We have shown that siRNAs may provide a novel approach to circumvent such resistance mechanisms. Treatment
of TS-overexpressing RKO cells with siRNA concentrations as low as 10 pm can reduce IC50 values by twofold, whereas a concentration of 1 nm TS1058 siRNA restored chemosensitivity to three different TS anticancer agents (ZD1694, 1843UB9, and FuUrd) by 15-fold.

In the present report, we show that siRNAs directed at TS mRNA specifically and potently repressed expression of TS mRNA and protein in human colon cancer RKO cells via the process of RNAi. This approach may prevent and/or overcome the acute induction of TS and the subsequent development of cellular resistance observed with TS inhibitor compounds now being used in the clinical setting. In this regard, the use of siRNA molecules may have therapeutic promise as a strategy to be used alone or in combination with other established TS inhibitor compounds. Moreover, these studies may provide new insights as to how siRNAs directed at other critical signaling pathways may be developed as novel therapeutic molecules for the treatment of human cancer.

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REFERENCES

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