Combining Small Interfering RNAs Targeting Thymidylate Synthase and Thymidine Kinase 1 or 2 Sensitizes Human Tumor Cells to 5-Fluorodeoxyuridine and Pemetrexed

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ABSTRACT
Thymidylate synthase (TS) is the only de novo source of thymidylate (dTMP) for DNA synthesis and repair. Drugs targeting TS protein are a mainstay in cancer treatment, but off-target effects and toxicity limit their use. Cytosolic thymidine kinase (TK1) and mitochondrial thymidine kinase (TK2) contribute to an alternative dTMP-producing pathway, by salvaging thymidine from the tumor milieu, and may modulate resistance to TS-targeting drugs. Combined down-regulation of these enzymes is an attractive strategy to enhance cancer therapy. We have shown previously that antisense-targeting TS enhanced tumor cell sensitivity to TS-targeting anticancer drugs. We assessed the effects of targeting TK1 or TK2 with siRNA alone and in combination with siRNA targeting TS and/or TS-protein targeting drugs on tumor cell proliferation. Down-regulation of TK with siRNA enhanced the capacity of TS siRNA to sensitize tumor cells to TS-targeting drugs [5-fluorodeoxyuridine (5FUdR) and pemetrexed]. The sensitization was greater than that observed in response to any siRNA used alone and was specific to drugs targeting TS. Up-regulation of TK1 in response to combined 5FUdR and TS siRNA suggests that TK knockdown may be therapeutically useful in combination with these agents. TKs may be useful targets for cancer therapy when combined with molecules targeting TS mRNA and TS protein.

Introduction
Deoxy-TTP, required for DNA synthesis and repair, is synthesized by two different pathways in mammalian cells (Hu and Chang, 2007). De novo synthesis is mediated by thymidylate synthase (TS), which catalyzes the reductive methylation of dUMP to dTMP using $N_\text{5}$-$N_\text{10}$-methylene-tetrahydrofolate as the methyl donor (Carreras and Santi, 1995). Salvage synthesis is mediated by thymidine kinases (TKs), which produce dTMP by phosphorylation of deoxythymidine obtained from the tumor environment.

TS enzyme levels are cell cycle-dependent, peaking during the S phase and decreasing before $G_2$ (Nagarajan and Johnson, 1989). Proliferating cells (including cancer cells) have higher levels of TS than nonproliferating cells (Derenzini et al., 2002). TS has been an important drug target for cancer treatment for more than 50 years (Douglas, 1987; Costi, 1998). Fluorinated pyrimidines, including 5-fluorouracil (5FU) and its metabolite 5-fluorodeoxyuridine (5FUdR), inhibit TS by mimicking dUMP and irreversibly binding to the enzyme (Heidelberger et al., 1957; Berg et al., 2002). Folate analogs such as pemetrexed block the capacity of TS to associate with $N_\text{5}$-$N_\text{10}$-methylene-tetrahydrofolate (Chu et al., 2003).

The usefulness of TS-targeting drugs in cancer treatment is limited by toxicity to nontumor tissues and inherent drug resistance of some tumor types. Resistance can be caused by increased TS levels (even in the presence of a TS inhibitor) mediated by: 1) TS gene amplification (Berg et al., 2002), 2) translational derepression (i.e., reduced capacity of TS
protein to associate with its own mRNA to repress translation) (Schmitz et al., 2001), and/or 3) TS enzyme stabilization (Kitchens et al., 1999). In addition, the need for de novo dTMP production by TS can be lessened by TK-mediated phosphorylation of thymidine.

There are two types of TK: cytosolic TK1 and mitochondrial TK2. TK1 and TK2 are distinct proteins encoded by genes located on different chromosomes (Arné and Eriksson, 1995; Pérez-Pérez et al., 2008). TK2 is constitutively expressed at low levels throughout the cell cycle, whereas TK1 expression is cell cycle-dependent and highest in the S phase. TK1 levels are increased in proliferating cells (Arné and Eriksson, 1995; Luo et al., 2010).

Elevated serum TK (95% of which is TK1) is associated with poor prognosis in several human cancers including chronic lymphocytic leukemia, other blood cancers, and ovarian, breast, prostate, and colorectal cancers (Fujiwaki et al., 2002; Hamblin, 2007; Svobodova et al., 2007). Because both TKs and TS generate dTMP, TKs may compensate for reduced dTMP after treatment with TS-targeting drugs. There are currently no pharmacological agents that inhibit mammalian TK for cancer therapy. Given the current limitations of TS-targeting drugs, RNA interference (RNAi) targeting TKs [alone or in combination with small interfering RNA (siRNAs) targeting TS] is an attractive tool to both explore TK function in the context of TS activity and increase the effectiveness of TS-targeting drugs.

RNAi molecules [siRNA, oligodeoxynucleotides (ODNs), microRNA, and short-hairpin RNA] are powerful tools for regulating and exploring gene expression and are emerging as agents for treating human disease (Aigner, 2007). RNAi can inhibit translation of specific mRNAs and/or target those mRNAs for destruction by sequence-specific cleavage and degradation (Castanotto and Rossi, 2009). Exogenous RNAi molecules have potential as therapeutic agents for treating multiple human diseases including cancer (Behlke, 2006; Lares et al., 2010). There are currently many ODNs and siRNAs in clinical trials for the treatment of different cancers, viral infections, and other diseases and pathological conditions (Lares et al., 2010). With respect to cancer, these agents can modify molecules upon which tumor cells rely and act in combination with current small-molecule anticancer drugs to enhance treatment (Di Cresce and Koropatkин, 2010; Kanwar et al., 2010).

We, and others, have demonstrated that ODNs targeting TS sensitize human tumor cells (and resensitize 5FuDr-resistant human tumor cells) to the cytotoxic effects of 5FuDr and other TS-targeting drugs both in vitro and in vivo (Ferguson et al., 1999, 2001; Berg et al., 2001). We also explored the potential benefit of simultaneously targeting two mRNAs (encoding Bcl-2 and TS) that mediate resistance to chemotherapy. Although TS siRNA sensitized tumor cells to TS-targeting drugs (similar to antisense ODNs), combined Bcl-2 siRNA and TS siRNA produced nonreciprocal antagonism that reduced the effectiveness of TS siRNA, for reasons that could not be attributed to siRNA complementarity (Castanotto et al., 2007; Pandyra et al., 2007). Combinatorial RNAi approaches require careful evaluation before application.

Because both TS and TKs generate dTMP, it is possible that TKs mediate resistance to TS-targeting drugs and potentially to TS siRNA. We assessed the effects of targeting TK1 or TK2 with siRNA (alone and in combination with siRNA targeting TS and/or TS protein-targeting drugs) on tumor cell proliferation. We showed that down-regulation of TK with siRNA enhances the capacity of TS siRNA to sensitize tumor cells to traditional TS protein-targeting drugs (5FuDr and pemetrexed), but not to an anticancer drug that does not target TS (cisplatin).

### Materials and Methods

#### Human Tumor Cell Lines

Human cervical carcinoma (HeLa) and breast epithelial adenocarcinoma (MCF7) cell lines were purchased from the American Type Culture Collection (Manassas, VA). HeLa and MCF7 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Wisent Biotech, St-Bruno, QC, Canada) with 10% fetal bovine serum (FBS; Invitrogen, Burlington, ON, Canada). All cell lines were maintained in a humidified incubator at 37°C with 5% CO2 in air.

#### siRNAs

All siRNAs (ON-TARGET plus or siGENOME) were obtained from Dharmacon RNA Technologies (Lafayette, CO) as annealed and desalted duplexes. ON-TARGET plus siRNAs contain a chemical modification that enhances siRNA antisense strand entry into the RNA-induced silencing complex for certain sequences (identified by Dharmacon RNA Technologies’s siRNA design algorithm) where the modification is likely to enhance specific activity without increasing off-target binding (Khvorova et al., 2003; Birmingham et al., 2006; Jackson et al., 2006; Anderson et al., 2008; Chen et al., 2008). ON-TARGET plus or siGENOME reagents were used as recommended by Dharmacon RNA Technologies for each targeted mRNA sequence (Table 1). All siRNAs were dissolved in siRNA buffer (supplied by Dharmacon RNA Technologies) to generate 5 or 10 μM stock solutions.

#### Cytotoxic Drugs

Pemetrexed was purchased from Sigma-Aldrich (St. Louis, MO). Pemetrexed (Alimta, manufactured by Eli Lilly and Co., Toronto, ON, Canada) and cisplatin (Platinsol, manufactured by Bristol-Myers Squibb, Montreal, QC, Canada) were obtained from the pharmacy at University of British Columbia (Vancouver, BC, Canada). (Ferguson et al., 1999, 2001; Berg et al., 2001).

### Table 1

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Targeted RNA</th>
<th>Target mRNA Sequence</th>
<th>Position in mRNA Transcript</th>
</tr>
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<tbody>
<tr>
<td>ON-TARGETplus</td>
<td>Nontargeting</td>
<td>No target</td>
<td>5'-GCGUUGUAGAAGGCUAAG-3'</td>
</tr>
<tr>
<td>ON-TARGETplus</td>
<td>Nontargeting</td>
<td>No target</td>
<td>5'-GCGUUGUAGAAGGCUAAG-3'</td>
</tr>
<tr>
<td>TYMS siGENOME</td>
<td>TS siRNA(1)</td>
<td>TS mRNA</td>
<td>5'-GACAGAAGAAGAAGAAGA-3'</td>
</tr>
<tr>
<td>TYMS siGENOME</td>
<td>TS siRNA(2)</td>
<td>TS mRNA</td>
<td>5'-GACAGAAGAAGAAGAAGA-3'</td>
</tr>
<tr>
<td>Human TK1 ON-TARGETplus</td>
<td>siRNA, TK1 siRNA(1)</td>
<td>TK1 mRNA</td>
<td>5'-CAGAGACACGCGCGCGCGCGA-3'</td>
</tr>
<tr>
<td>Human TK1 ON-TARGETplus</td>
<td>siRNA, TK1 siRNA(2)</td>
<td>TK1 mRNA</td>
<td>5'-CAGAGACACGCGCGCGCGCGA-3'</td>
</tr>
<tr>
<td>Human TK2 ON-TARGETplus</td>
<td>siRNA, TK2 siRNA(1)</td>
<td>TK2 mRNA</td>
<td>5'-CAAGACACGCGCGCGCGCGA-3'</td>
</tr>
<tr>
<td>Human TK2 ON-TARGETplus</td>
<td>siRNA, TK2 siRNA(2)</td>
<td>TK2 mRNA</td>
<td>5'-CAAGACACGCGCGCGCGCGA-3'</td>
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Control siRNAs (C2, C3) contain four or more mismatches with all known human RNAs.
siRNA Transfection

Twenty-four hours before siRNA transfection cells were plated in 2 ml of DMEM with 10% FBS in NUNC 25-cm² tissue culture flasks (T-25, Nunclon ∆ surface) (VWR International, Ontario, Canada) as follows: HeLa, 1.5 × 10⁶ cells per flask; MCF7, 2.0 × 10⁶ cells per flask. Cells were transfected with one siRNA or a mixture of two siRNAs using Lipofectamine 2000 reagent (LF2K; Invitrogen, Carlsbad, CA) or treated with LF2K alone. Transfections were performed according to a modification of the manufacturer’s protocol that maximized transfection efficiency but reduced the amount of LF2K required by 50%. In brief, LF2K mixture (120 μl of serum-free DMEM and 5 μl of LF2K) was mixed with siRNA (in serum-free DMEM, sufficient to result in a final total siRNA concentration of 10 nM when added to cells), or in some control experiments DMEM alone was used. The mixture was added to 2 ml of media in each T25 flask. Total siRNA concentrations for each transfection were held constant (10 nM) by combining two targeting siRNAs (5 nM each) or, when a single targeting siRNA was used, combining 5 nM targeting siRNA plus 5 nM nontargeting control 2 siRNA (C2). Cell culture medium (4 ml, DMEM with 10% FBS) was added to flasks 4 h after the addition of siRNAs and/or LF2K. Twenty hours later (i.e., 24 h post-siRNA addition) cells were collected for RNA isolation and analysis of TS, TK1, and TK2 mRNA levels. Cells were also collected at 48 h post-siRNA addition for protein immunoblot analysis.

Modified siRNA Transfection for Assay of Sensitivity to Cytotoxic Drugs

To obtain sufficient numbers of siRNA-transfected cells for cytotoxicity assays, maintain constant transfection conditions, and minimize potential differences induced by independent transfections, HeLa cells were first plated in T25 flasks in 2 ml of DMEM (4.0 × 10⁶ cells per flask). Cells were transfected 24 h later with siRNAs or LF2K alone (three flasks per condition) as described above. Four hours after the addition of siRNAs or LF2K, twenty hours later (i.e., 24 h post-siRNA addition) cells were collected for RNA isolation and analysis of TS, TK1, and TK2 mRNA levels. Cells were also collected at 48 h post-siRNA addition for protein immunoblot analysis.

Measurement of TS, TK1, and TK2 mRNA

Cells collected for mRNA analysis were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA), and total cellular RNA was isolated according to the manufacturer’s protocol. Purified RNA (1 μg) was used to synthesize cDNA by reverse transcription mediated by Moloney murine leukemia virus-reverse transcriptase (Invitrogen) and random primers according to the protocol provided by the manufacturer. TS, TK1, and TK2 mRNA levels and 18S rRNA levels were assessed simultaneously by multiplex real-time polymerase chain reaction amplification using a TaqMan Gene Expression Assay kit (Applied Biosystems, Foster City, CA) and specific primers and probes (Table 2). TS, TK1, and TK2 mRNA levels were expressed relative to 18S rRNA levels.

Measurement of TS and Actin Immunoblots

Total cell protein lysates were obtained at the indicated times. Cells were washed twice with ice-cold PBS, resuspended in cell lysis buffer (1 M Tris, pH 7.6, 0.1% SDS, 1% Triton X-100, 0.2 M EDTA), and sonicated at 4°C to disrupt membranes. Protein concentration was estimated using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Quebec, Canada) using the protocol provided by the manufacturer. Cell lysates (25 μg of total soluble protein per lane when assessing TS and actin protein; 40 μg of total soluble protein per lane when assessing TK1 or TK2 and actin) were resolved by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) and transferred to a nitrocellulose membrane (Hybond-ECL, GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Membranes were blocked for 1 h [5% milk in TBS-T; TBS plus Tween 20 (0.2%)] and then washed with fresh TBS-T. Bands were detected and quantified as described below.

Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>TaqMan Probe</th>
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<tbody>
<tr>
<td>TS</td>
<td>5′-GCCCCTGCCCCCTGGCTCT-3′</td>
<td>5′-GATGCTGCGCAGCTATACAT-3′</td>
<td>FAM-AACTCGCCACCATCACTCCCTC-5′–MGB/NFQ</td>
</tr>
<tr>
<td>TK1</td>
<td>5′-TCCCTCAACCCCTCGTCTTCC-3′</td>
<td>5′-TGCGACCCATCCTGCTGGTA-3′</td>
<td>NED-ACGACCACGACGGAC-MGB/NFQ</td>
</tr>
<tr>
<td>TK2</td>
<td>5′-CGCCGAGAGCGCAACCGTCT-3′</td>
<td>5′-TCTCTCAAGAACCAACAGCCCACACGT-3′</td>
<td>FAM-CACCGAGAATCATCC-5′–MGB/NFQ</td>
</tr>
<tr>
<td>r18S</td>
<td>As supplied</td>
<td>As supplied</td>
<td>VIC-labeled</td>
</tr>
</tbody>
</table>

Proliferation

The effects of siRNA treatment, alone or in combination with anticancer drugs, on cell proliferation were assessed. Cells exposed to various treatments were grown for 4 days, washed with PBS, trypsinized, and counted on a Beckman Coulter Z1 Particle Counter (Beckman, Mississauga, ON, Canada). Raw cell counts were analyzed by calculating the fold change in cell number after 4 days of growth (relative to the starting number of plated cells) as:

\[
\text{Fold change} = \left( \frac{\text{number of cells, day 4}}{\text{number of cells, day 0}} \right)
\]

Differences in proliferation induced by treatment were expressed as percentage of fold change and calculated using fold change in cell number in treated cells (treatment fold change) and the fold change in cell number under appropriate matched control conditions (control fold change):

\[
\% \text{ Fold change} = \left( \frac{\text{treatment fold change}}{\text{control fold change}} \right) \times 100
\]
and incubated with horseradish peroxidase-conjugated α-rabbit IgG antibody (1:3000 in TBS-T/1% skim milk, 1 h, 20°C). Bands were detected and quantified as described below. The membrane was then stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA; 15 min, 20°C), washed with TBS-T, and cut into two pieces (>37 and <37 kDa) using the Kaleidoscope Precision Plus Protein Standards Ladder (Bio-Rad Laboratories) as a guide. The <37-kDa membrane was incubated with mouse polyclonal anti-human TK1 antibody (QED Bioscience, San Diego, CA; 1:400 in TBS-T/1% skim milk, 1 h, 20°C) and the >37-kDa membrane was incubated with α-actin antibody (1:1000 in TBS-T/1% skim milk, 1 h, 20°C) and washed with TBS-T. Membranes were then incubated with either horseradish peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare; 1:3000 in TBS-T/1% skim milk, 1 h, 20°C) for TK1 detection or α-rabbit antibody (1:3000 in TBS-T/1% skim milk, 1 h, 20°C) for TK2 detection.

**Immunoblot Band Detection and Quantification.** Horseradish peroxidase activity associated with protein bands was detected using Enhanced Chemiluminescence Plus (ECL Plus; GE Healthcare) and a STORM 860 Molecular Imager (phosphoimager/fluorimager) (Molecular Dynamics, Sunnyvale, CA). Band intensity was quantified using ImageQuant 5.1 software (Molecular Dynamics).

**Statistical Analysis**
Data are presented as means ± S.E.M. To determine the significance of differences between means, ANOVA (when measuring differences among multiple means) or Student’s t tests (when measuring differences between two means) were performed. The level of significance for all statistical analyses was chosen a priori to be *p* < 0.05.

**Results**

**TS and TK siRNAs Decreased Target mRNAs in HeLa and MCF7 Cells.** Two different siRNAs targeting TS (TS siRNA(1) and TS siRNA(2)) were evaluated. TS siRNA(1) and TS siRNA(2) each down-regulated TS mRNA by 70 to 85% in HeLa (Fig. 1A) and MCF7 (Fig. 1D) cells. The capacity of either TS siRNA to down-regulate TS was unaffected by...
simultaneously targeting TK1 or TK2 (in combination with TS siRNA) using two different TK siRNAs in both HeLa (Fig. 1A) and MCF7 (Fig. 1D) cells.

Likewise, two different siRNAs targeting TK1 [TK1 siRNA(1) and TK1 siRNA(2)] down-regulated TK1 mRNA by 60 to 80% in HeLa (Fig. 1B) and MCF7 (Fig. 1E) cells. In MCF7 (but not HeLa) cells, TK1 siRNA(1) down-regulated TK1 more effectively than TK1 siRNA(2). The capacity of either of the two TK1 siRNAs to down-regulate TK1 was unaffected by simultaneous administration of TS siRNA and both were effective down-regulators of TK1 mRNA in HeLa (Fig. 1B) and MCF7 (Fig. 1E) cells.

TK2 was also susceptible to siRNAs, although to a more variable degree than TS and TK1. Two different siRNAs (TK2 siRNA(1) and TK2 siRNA(2)) down-regulated TK2 by more than 80% in HeLa cells (Fig. 1C) and 45 to 50% in MCF7 cells (Fig. 1D). TK2 siRNA(1) was more effective than TK2 siRNA(2) in both HeLa (Fig. 1C) and MCF7 (Fig. 1F) cells. Similar to the situation with TK1 siRNAs, combined treatment with TK2 and TS siRNAs did not alter the effectiveness of TK2 siRNAs (Fig. 1, C and F).

Cell lines were treated with the lowest siRNA and LF2K concentrations possible to maximize knockdown of target mRNAs, minimize nonspecific effects, and yield consistent target down-regulation in replicate experiments (optimization data not shown). Total siRNA concentrations were held constant (10 nM) by adding nontargeting C2 siRNA to siRNAs targeting TS, TK1, or TK2 where necessary, a maneuver that did not alter the effectiveness of siRNAs (data not shown). These preliminary experiments tested two different targeting siRNAs for each target mRNA (TS, TK1, and TK2) in HeLa and MCF7 cell lines. Results were obtained 24 h post-transfection.

TS siRNA(2), TK1 siRNA(2), and TK2 siRNA(1) were the most effective among the six siRNAs tested and were selected for use in all subsequent experiments (Figs. 2–10).

Combined Treatment with TS and TK2 siRNAs Sensitized HeLa Cells to 5FUdR Better than TS siRNA Alone. To test the capacity of combined reduction of both TS and TKs to enhance the activity of a TS-targeting drug, we measured the effect of siRNA down-regulation of both TS and TKs alone, and in combination, followed by a period of treatment with or without 5FUdR, on HeLa cell proliferation. Results were obtained 96 h post-transfection and/or treatment with drug.

As single agents, siRNAs targeting TS, TK1, and TK2 had no effect on HeLa cell proliferation compared with treatment with control, nontargeting siRNA (Fig. 2A). Treatment with 5FUdR (IC50), without knockdown of TS, TK1, or TK2, reduced proliferation by approximately 50%, as expected. Pretreatment with specific siRNA to knockdown TK1 or TK2 before 5FUdR treatment did not enhance the antiproliferative effect of 5FUdR (Fig. 2A). siRNA knockdown of TS before 5FUdR treatment enhanced the antiproliferative effect of 5FUdR by approximately 50%, as reported previously (Ferguson et al., 1999, 2001; Flynn et al., 2006; Pandyra et al., 2007). However, when both TS and TK2 mRNA were decreased by siRNA before 5FUdR treatment (see Figs. 3 and 5), proliferation was reduced by approximately 20% more than after 5FUdR and knockdown of TS alone (an overall decrease of 60% compared with 50% when each was compared with control) (Fig. 2). The capacity of TK knockdown to increase TS siRNA-mediated enhancement of the antiproliferative effect of 5FUdR was specific to TK2. siRNA knockdown of TK1 had no effect on TS siRNA enhancement of 5FUdR activity (Fig. 2).

siRNA Down-Regulation of TS, TK1, or TK2 Persisted up to 96 H Post-Transfection, with and without 5FUdR Treatment. TS siRNA (Fig. 3), TK1 siRNA (Fig. 4), and TK2 siRNA (Fig. 5) were applied to HeLa cells in three different protocols: 1) as single agents, 2) TS siRNA combined with TK1 siRNA or TK2 siRNA, and 3) followed by treatment with 5FUdR. All siRNAs down-regulated their target mRNAs, regardless of protocol, up to 96 h post-transfection. The degree of mRNA down-regulation at 96 h was less than that at 24 h post-transfection (Fig. 1). At 96 h after treatment with TS siRNA alone, TS mRNA levels were 28% of control (Fig. 3A) compared with 7% of control at 24 h (Fig. 1A). At 96 h after TK1 siRNA treatment alone, TK1 mRNA was 52% of control (Fig. 4A) compared with 10% of control at 24 h (Fig. 1A). At 96 h after TK2 siRNA treatment alone, TK2 mRNA...
was 75% of control (Fig. 5A) compared with 16% of control at 24 h (Fig. 1A). Likewise, time-dependent attenuation of target mRNA was evident under conditions where TS siRNA was combined with TK1 siRNA or TK2 siRNA, with or without 5FUdR.

All siRNAs decreased target protein levels at 96 h post-transfection. TS siRNA alone decreased TS protein by 70% (Fig. 3C), TK1 siRNA alone reduced TK1 protein by 57% (Fig. 4C), and TK2 siRNA alone reduced TK2 protein by 30% (Fig. 5C). Likewise, siRNA-mediated reduction in levels of pro-
proteins translated from targeted mRNAs was observed under conditions where TS siRNA was combined with TK1 siRNA or TK2 siRNA, with or without 5FUdR (Figs. 3–5).

**TS siRNA Plus 5FUdR Increased TK1 Protein.** TS siRNA, alone or combined with TK2 siRNA, followed by 5FUdR, increased TK1 protein levels. TS siRNA plus 5FUdR increased TK1 protein 2-fold (Fig. 4C) compared with the level seen after treatment with TS siRNA alone (Fig. 4B). The increase was not apparently caused by increased TK1 mRNA (Fig. 4A) and was abolished when TK1 siRNA was combined with TS siRNA before 5FUdR treatment (Fig. 4C). TS siRNA treatment, alone or in combination with TK1, TK2, and/or 5FUdR, did not increase TK2 protein (Fig. 5, B and C).

**Combined Treatment with TS and TK1 siRNAs Sensitized HeLa Cells to Pemetrexed Better than TS siRNA Alone.** HeLa cells were treated with siRNAs and pemetrexed (as described above for combined treatment with 5FUdR), and the effects on proliferation were evaluated 96 h later. TS siRNA, combined or not with TK1 or TK2 siRNAs, had no effect on proliferation without pemetrexed (compared with treatment with C2 siRNA, Fig. 6A). Treatment with pemetrexed (IC50) reduced proliferation in all cases. TS siRNA treatment, alone or in combination with TK1, TK2, and/or 5FUdR, did not increase TK2 protein (Fig. 5, B and C).

**Discussion**

Both thymidylate synthase and thymidine kinases mediate production of dTMP for DNA synthesis and repair. The activity of both pathways increases intracellular dTMP and can potentially contribute to tumor cell resistance to drugs targeting TS. We hypothesized that, when TS is inhibited by anti-TS drugs and/or TS siRNA, TKs are important in mediating resistance to those agents. Consequently, and in addition, we hypothesized that siRNA targeting TK alone or in combination with TS siRNA increased the antiproliferative effect of pemetrexed by approximately 55% compared with C2 siRNA.

siRNA Down-Regulation of TS, TK1, or TK2 Persists Up to 96 H Post-Transfection, with and without Pemetrexed Treatment. As seen after combined treatment with siRNAs targeting TS or TS plus TK1 or TK2, with or without subsequent 5FUdR (Figs. 3–5), siRNA-mediated down-regulation of mRNA and protein targets was maintained up to 96 h post-transfection of HeLa cells and in the presence of pemetrexed (TS, Fig. 7; TK1, Fig. 8; TK2, Fig. 9). Control data presented in Figs. 3 to 5 were generated from similar experiments, shown in each case because they were performed simultaneously with and controlled for potential variation in interexperiment culture conditions and cell characteristics. Data shown in Figs. 5B and 9B are identical and shown in each case for ease of comparison.

TS siRNA plus pemetrexed increased TK2 protein to a level 70% higher than that observed after treatment with TS siRNA alone (Fig. 9, B and C). This effect was negated by the presence of TK2 siRNA. There was no concomitant increase in TK1 protein (Fig. 8B).

**siRNA-Mediated Reduction of TS and TKs Does Not Enhance Cytotoxicity of Cisplatin.** Enhancement of drug-mediated antiproliferative effects by siRNA knockdown of TS and TK in HeLa cells was specific to 5FUdR and pemetrexed. TS and TK siRNAs, alone or in combination, had no effect on cisplatin-mediated inhibition of proliferation (Fig. 10).
To test these hypotheses, we treated HeLa and MCF7 cells with siRNAs targeting TS alone, TK1 or TK2 alone, or TS and TK2 siRNA, in various combinations as described under Materials and Methods, followed by pemetrexed (IC_{50}) (filled bars) or no drug (empty bars) for 96 h. Proliferation was measured as described under Materials and Methods. B, proliferation of cells treated with pemetrexed plus TS siRNA, with or without TK1 siRNA. Proliferation of cells untreated with drug or siRNAs, and cells treated with various siRNAs alone, was calculated as a percentage of cells treated with control (C2) siRNA only. Proliferation of cells treated with pemetrexed was calculated as a percentage of identically treated cells without pemetrexed. NTC and LF2K data are included to show that transfection conditions had minimal effects on proliferation. Bars represent means ± S.E. (three independent experiments; n = three for each treatment group in each experiment). * indicates different from cells treated with control (C2) siRNA (p < 0.05, ANOVA); a indicates different from cells treated with TS siRNA(1) plus C2 siRNA (p < 0.05, ANOVA and/or Student’s t test).

Fig. 6. Simultaneous treatment with TS and TK1 siRNAs sensitizes HeLa cells to pemetrexed better than treatment with TS siRNA alone. A, HeLa cells were treated with control siRNA, TS siRNA, TK1 siRNA, and TK2 siRNA, in various combinations as described under Materials and Methods, followed by pemetrexed (IC_{50}) (filled bars) or no drug (empty bars) for 96 h. Proliferation was measured as described under Materials and Methods. B, proliferation of cells treated with pemetrexed plus TS siRNA, with or without TK1 siRNA. Proliferation of cells untreated with drug or siRNAs, and cells treated with various siRNAs alone, was calculated as a percentage of cells treated with control (C2) siRNA only. Proliferation of cells treated with pemetrexed was calculated as a percentage of identically treated cells without pemetrexed. NTC and LF2K data are included to show that transfection conditions had minimal effects on proliferation. Bars represent means ± S.E. (three independent experiments; n = three for each treatment group in each experiment). * indicates different from cells treated with control (C2) siRNA (p < 0.05, ANOVA); a indicates different from cells treated with TS siRNA(1) plus C2 siRNA (p < 0.05, ANOVA and/or Student’s t test).

A combination with TS siRNA would further enhance the effectiveness of TS-targeting drugs.

To test these hypotheses, we treated HeLa and MCF7 cells with siRNAs targeting TS alone, TK1 or TK2 alone, or TS siRNA in combination with siRNA targeting TK1 or TK2. It was important to establish that simultaneous administration of siRNAs targeting different mRNAs did not affect the capacity of each siRNA capacity to lower the level of its target mRNA. Previous reports (by our group and others) have suggested that combining different siRNAs can have reciprocal and nonreciprocal inhibitory effects on each other’s activity caused by competition for entry into RNA-induced silencing complexes and/or direct base-pairing with each other because of short regions of complementarity (Birmingham et al., 2006; Castanotto et al., 2007; Pandyra et al., 2007). Each of the siRNAs used in this study down-regulated their target mRNA, alone or in combination. Targeting TS mRNA had no effect on TK1 or TK2 mRNA levels and vice versa (Fig. 1). All siRNAs were more potent in reducing target mRNA levels in HeLa than in MCF7 cells, and TS siRNAs were generally more effective than siRNAs targeting TK1 or TK2 (Fig. 1). The capacity of two different siRNAs to down-regulate each mRNA target was compared, and only the most potent was used for subsequent experiments in HeLa cells.

TS siRNA increased 5FUdR-mediated inhibition of HeLa cell growth by approximately 50% (Fig. 2A) as reported previously (Ferguson et al., 1999, 2001; Berg et al., 2001, 2002; Flynn et al., 2006; Pandyra et al., 2007). When TK2 siRNA was combined with TS siRNA, it increased sensitivity to 5FUdR by approximately 25% more than treatment with TS siRNA alone (Fig. 2). The phenomenon was isoform-specific: TK1 siRNA combined with TS siRNA did not add to the increase in drug sensitivity induced by TS knockdown. These data strongly suggest that at least one TK isoform (TK2) mediates sensitivity to 5FUdR. However, that participation seems to be indirect: siRNA-mediated knockdown of either TK2 or TK1 alone had no effect, under these experimental conditions, on 5FUdR sensitivity (Fig. 2A). This is consistent with a model in which TS-mediated synthesis of thymidylate is the predominant cellular source (which is the case under normal conditions; Arner and Eriksson, 1995), but where alternative TK-mediated production can partially compensate when de novo thymidylate synthesis is impaired by combined treatment with TS siRNA (to decrease TS mRNA) and 5FUdR (to decrease TS enzyme activity). Although our data provide evidence of a role only for TK2 with respect to 5FUdR sensitivity, we cannot exclude involvement of TK1 under different conditions. TK1 activity and expression is higher in proliferating cells compared with TK2 (Arner and Eriksson, 1995; Luo et al., 2009) and the degree of TK1 knockdown achievable using this technology may not be sufficient to enhance the effects of combined TS siRNA and 5FUdR.

We have reported that a threshold exists in antisense knockdown of TS to enhance sensitivity to TS-targeting drugs (Ferguson et al., 1999). We suggest that a similar threshold exists for TK1 and/or TK2, and that threshold may differ between the two. If so, a smaller decrease in TK2, compared with TK1, might be all that is required to induce a significant effect to 5FUdR under our experimental conditions. The converse may be true for pemetrexed. Finally, although we assessed the role of both TK1 and TK2 in combination with 5FUdR treatment at the IC_{50} concentration, the effect of TK1 down-regulation might be evident only in combination with higher 5FUdR concentrations that reduce TS activity and cell viability more profoundly. These possibilities are currently being investigated.

Issues such as those discussed above might be responsible for reports that targeting TK1 had no effect on sensitivity to TS-targeting drugs (Kinsella et al., 1997; Lee et al., 2010). Those studies may also have been limited by the use of 5FU (a less specific TS inhibitor than 5FUdR; Longley et al., 2003), the focus solely on TK1 (TK2 has not previously been assessed as a potential cancer therapeutic target), and the use of dipyridamole for global inhibition of nucleoside transporters rather than specific inhibition of thymidylate production. Early clinical trials aimed at disruption of the TK-mediated salvage pathway probably were limited by the available technology, method of administration, and bioavailability of dipyridamole (Buzaid et al., 1989; Pickard and
Kinsella, 1996; Kinsella et al., 1997). Regardless, the results suggested that thymidine salvage was an important factor in response to TS-targeting drugs and folate inhibitors.

TK siRNA-mediated enhancement of the increase in drug sensitivity induced by TS siRNA was not restricted to 5FUdR. Combined treatment with TK1 siRNA and TS siRNA enhanced sensitivity to pemetrexed (a multitargeted antifolate with a different mode of action than 5FUdR) by approximately 30% more than the enhancement induced by TS siRNA alone. Similar to TK effects on 5FUdR sensitivity, the
phenomenon was isoform-specific. In this case, however, enhancement was caused by TK1 siRNA and not TK2 siRNA (Fig. 6). Therefore, although our data show that TK plays a role in sensitivity to two different TS-targeting drugs, sensitivity to 5FUdR and pemetrexed in response to knockdown of each of the two isoforms is different, for reasons that are not yet clear. Although both pemetrexed and 5FUdR are TS inhibitors, their interactions with TS and other cellular targets are quite different. Pemetrexed, unlike 5FUdR, interacts with multiple folate-dependent enzymes required for production of DNA and RNA intermediates, including glycineamide ribonucleotide transerase and dihydrofolate reductase (Chattopadhyay et al., 2007). Differential roles for TK isoforms in connection with inhibition of individual or multiple folate-requiring enzymes have not yet been explored. In particular, TK1 is localized primarily in cytoplasm and TK2 in mitochondria (Arné and Eriksson, 1995), and the contribution of mitochondrial thymidine and folate metabolism to drug sensitivity is unknown. Our data suggest nonoverlapping functions of TK1 and TK2, perhaps based on cellular location, that require investigation.

An unexpected finding was that treatment with TS siRNA in combination with 5FUdR increased TK1 protein (Fig. 4C), and TS siRNA combined with pemetrexed increased TK2 protein (Fig. 9C). The increases were not apparently caused by increased gene transcription because they were evident in the absence of increased TK1 or TK2 mRNA (Figs. 4A and 9A). Whatever the mechanism, these increases in response to TS inhibition support the hypothesis that TKs could mediate enhanced cell survival (and, conversely, knockdown of TKs may reduce that survival in tumor cells) when TS activity is reduced. In support of this, induction of TK1 in response to TS inhibition by 5-FU (the prodrug of 5-FUdR) has been reported and invoked as a mechanism for increased uptake of 3'-deoxy-3'-[18F]fluorothymidine (the so-called “flare response”) (Lee et al., 2010). That report is consistent with our observation of increased TK1 after treatment with TS siRNA and 5FUdR. Our observation of a specific increase in TK2 protein in response to pemetrexed (indeed, to any TS-targeting drug) is novel, and we currently are investigating it further. However, the isoform-specific increase in TK1 and TK2 in response to 5FUdR and pemetrexed, respectively, is not fully consistent with our observation that siRNA-mediated knockdown of TK2 (but not TK1) contributes to 5-FUdR sensitivity and knockdown of TK1 (but not TK2) contributes to enhanced pemetrexed sensitivity. Experiments to further explore differential involvement of TK1 and TK2 in sensitivity to different classes of TS-targeting drugs (where those drugs are applied at concentrations other than the IC50 and TK1 and TK2 are overexpressed) are under way.

siRNA-mediated down-regulation of TS, alone or in combination with siRNA knockdown of TK1 or TK2, had no effect on sensitivity to cisplatin. This agrees with previous reports that antisense against TS increased tumor cell sensitivity

Fig. 9. TK2 siRNA reduces both TK2 mRNA and protein in HeLa cells, with or without simultaneous administration of TS siRNA and/or pemetrexed. HeLa cells were treated as described in the legend to Fig. 7. A, relative TK2 mRNA (percentage of that in cells treated with C2 siRNA); B and C, relative TK2 protein (percentage of that in cells treated with C2 siRNA) without (B) and with (C) pemetrexed. Representative immunoblots (one experiment per treatment condition) are shown (Ld, molecular mass marker; lane 1, untreated control; lane 2, LF2K; lane 3, C2; lane 4, C3; lane 5, TS + C2; lane 6, TK1 + C2; lane 7, TK2 + C2; lane 8, TS + TK1; lane 9, TS + TK2). Bars represent means ± S.E. (B, n = six independent experiments; C, n = three independent experiments). * indicates different from cells treated with control (C2) siRNA (p ≤ 0.05, ANOVA and/or Student's t test); a indicates different from cells treated identically but without pemetrexed (p ≤ 0.05, Student's t test).
Fig. 10. HeLa cell sensitivity to cisplatin is not affected by TS and/or TK siRNAs. HeLa cells were treated with control siRNA, TS siRNA, TK1 siRNA, and TK2 siRNA, in various combinations as described under Materials and Methods. Proliferation of cultures untreated with drug or siRNAs, and cells treated with various siRNAs alone, was calculated as a percentage of percent of percent of cells with treated with control (C2) siRNA only. Proliferation of cells treated with cisplatin was calculated as a percentage of identically treated cells without cisplatin. NTC and LFC2 DNA data are included to show that transfection conditions had minimal effects on proliferation. Bars represent means ± S.E. (three independent experiments; n = three for each treatment group in each experiment).

specifically to TS-targeting drugs but not to chemotherapeutics that do not target TS (Flynn et al., 2006; Pandyra et al., 2007) and supports the hypothesis that modulation of TK activity affects sensitivity only to anti-TS drugs.

Overall, these data support the hypothesis that TK enzymes are capable of reducing cellular sensitivity to TS-targeting drugs when the drugs are administered in combination with antisense molecules (siRNA) against TS. We demonstrated, for the first time, that a combinatorial RNAi approach (TS siRNA plus TK siRNA) enhanced human tumor cell sensitivity to two different TS-targeting drugs, and did so significantly more effectively than TS siRNA alone. In addition, these data revealed a novel distinction between TK1 and TK2 in their roles in sensitivity to TS-targeting drugs with different modes of action. We conclude that TK1 and TK2 are potential therapeutic targets to enhance tumor sensitivity to TS-targeting drugs with isofor specificity for certain classes of drug.

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Anti-human TS antibody was generously provided by Dr. Masakazu Fukushima (Taiho Pharmaceuticals, Hanno Research Center, Hanno, City, Japan).

Authorship Contributions

Participated in research design: Di Cresce, Figueredo, Ferguson, and Koropatnick.

Conducted experiments: Di Cresce.

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Luo P, Wang N, He E, Eriksson S, Zhou J, Hu G, Zhang J, and Skog S (2010) The proliferation marker thymidine kinase 1 level is high in normal kidney tubule cells treated with cisplatin was calculated as a percentage of identically treated cells without cisplatin. NTC and LFC2 data are included to show that transfection conditions had minimal effects on proliferation. Bars represent means ± S.E. (three independent experiments; n = three for each treatment group in each experiment).

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References


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